Characterization of Colon Cancer Cell Culture Based Screening Assay to Study Effects of Phenolic Acids

A Thesis Submitted to
the College of Graduate Studies and Research
in Partial Fulfillment of the Requirements
for the Degree of Master of Science
in the Department of Pathology and Laboratory Medicine
University of Saskatchewan
Saskatoon

By
Siew Hon Ng

PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements of a Postgraduate degree from the University of Saskatchewan, I agree that the Libraries of this University may make it freely available for inspection. I further agree that permission for copying of this thesis in any manner, in whole or in part, for scholarly purposes may be granted by the professor or professors who supervised this thesis work or, in their absence, by the Head of the Department or the Dean of the College in which this thesis work was done. It is understood that any copying or publication or use of this thesis or part thereof for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to the author and to the University of Saskatchewan in any scholarly use which may be made of any material in this thesis.

Requests for permission to copy or to make either use of the material presented in this thesis in while or part should be addressed to:

Head of the Department of Pathology and Laboratory Medicine

University of Saskatchewan

Saskatoon, Saskatchewan

S7N 0W8 CANADA

ABSTRACT

In Canada, colorectal cancer is the second leading cause of death from cancer in men and the third leading cause of death from cancer in women. Several factors contribute to the development of cancer. Genetic predisposition, diet, and lifestyle habits are some of the major factors for colorectal cancer development. In the diet related factors, epidemiological studies suggest that consumption of whole grains rich in dietary fiber are associated with low incidence of human colon cancer. Recent studies have also shown that, in addition to dietary fiber, the type of dietary fiber and other compounds such as phenolic acids present in cereal grain bran may also have a role to play in colon cancer prevention. In a recent study, eleven major phenolic acids which differed in anti-oxidant activity were identified in wheat bran from wheat varieties belonging to six different market classes. The main objective of this study was to develop an *in vitro* cell culture based assay system to study the effect of phenolic acids on colon cancer development. Another objective was to study the effect of phenolic acids on selected molecular markers associated with cell proliferation, apoptosis and inflammation. Two well established colon carcinoma cell lines HT-29 and HCT 116 were treated with varying concentrations of fourteen phenolic acids to study their effect on cell survival and proliferation. In addition, immunohistochemical assays were performed on treated cells for two cell proliferation markers (Cyclin D1 and Ki67), an apoptosis marker (Bax) and three inflammatory markers (Beta-catenin, COX-2 and iNOS). Treatment of phenolic acids inhibited the growth of both the cell lines, however the effects varied with phenolic acid and cell line used in the assay. As determined by IC_{50}, the growth of HCT 116 cells was inhibited the most by caffeic, ellagic, and gallic acids with IC_{50} of 0.22 mM, 0.17 mM,

and 0.15 mM, respectively. On the other hand, caffeic, chlorogenic, and gallic acids are most effective in preventing the growth of HT-29 cells, with IC_{50} at 0.06 mM, 0.28 mM, and 0.30 mM, respectively. Immunohistochemical and Western Blotting studies revealed that phenolic acids differentially affected markers for cell proliferation, apoptosis and cell inflammation. In most of the cases, phenolic acid treatments up-regulated the pro-apoptosis marker Bax, while it down-regulated cell proliferation marker Cyclin D1. The results clearly show that a cell culture based assay can be used to study the effect of phenolic acids or other chemical constituents isolated from plants to study their effect on colon cancer cell lines. Statistical analysis revealed that only in very limited cases, results of molecular markers correlated to cell growth and proliferation. Therefore, to draw firm conclusions, more detailed and extensive studied need to be completed using different phenolic acids, the two cell lines and more replications. However, this study has developed the necessary protocols and provided some indicative results such as most of the phenolic acids induced pro-apoptosis pathway in both the colon cancer lines. Future studies with extracted phenolic acids from wheat bran using the cell culture system optimized in this study can be used to define the role of different wheat varieties in colon cancer prevention.

ACKNOWLEDGEMENTS

I would like to take this opportunity to thank the people who have supported me during my Master's studies.

First of all, I would like to thank my supervisors, Drs. Rajni Chibbar and Ravi Chibbar. It is hard to overstate my gratitude to both my supervisors. Throughout my master's program and thesis-writing period, they provided encouragement, sound advice, good accompany and lots of brilliant ideas. They are the best professors I have ever met. This thesis will never be possible without them.

I greatly appreciate all the assistance provided by my advisory committee members: Drs. Rani Kanthan, Selliah Kanthan, John Krahn, Qing Meng, Mabood Qureshi and Joseph Blondeau.

I would also like to thank Ms. Heather Neufeld and Dr. Sarita Jaiswal for their professional expertise, advice and help in many different ways.

I would like to thank my boyfriend, Ze Long Lim for supporting and accompanying me. He keeps me motivated through tough times.

I am very grateful to my parents for supporting me both financially and emotionally. Without them I would never have the opportunity to study in Canada and to have this amazing experience.

This project was funded by the Canada Research Chairs (CRC), Natural Sciences and Engineering Research Council of Canada (NSERC), and Canada Foundation for Innovation (CFI) program.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ABTS	2, 2’-Azinobis(3-ethylbenzthiazoline-6-sulfonic acid)
AG	Aminoguanidine
Apaf-1	Apoptosis protease-activating factor-1
APC	Adenomatous polyposis coli
ATP	Adenosine triphosphate
AOM	Azoxymethane
Bak	Bcl-2 homologous antagonist killer
Bax	Bcl-2-associated X
Bcl-2	B-cell lymphoma 2
Bcl-xL	B-cell lymphoma-extra large
Caspase	Cysteinyl aspartate-specific protease
CDK	Cyclin dependent kinase
CHRPE	Congenital hypertrophy of the retinal pigment epithelium
CIN	Chromosomal instability
CK1	Casein kinase 1
cNOS	Constitutive nitric oxide synthase
COX-2	Cyclooxygenase-2
DCC	Deleted in colon cancer
DMEM	Dulbecco's modified Eagle's medium
DMH	Dimethylhydrazine
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
D-PBS	Dulbecco’s phosphate-buffered saline
DPPH	2, 2-Diphenyl-1-picrylhydrazyl
DSH	Dishevelled
ecNOS	Endothelial constitutive nitric oxide synthase
EDTA	Ethylenediaminetetraacetic acid
EP_2	E-prostanoid-2
FAP	Familial adenomatous polyposis
GLP-2	Growth hormone including peptide-2

GSK-3β	Glycogen synthase kinase-3β
H_2O_2	Hydrogen peroxide
HNPCC	Hereditary nonpolyposis colorectal cancer
IC_{50}	Half maximal inhibitory concentration
IGF	Insulin-like growth factor
iNOS	Inducible nitric oxide synthase
L-NAME	1-N(G)-nitroarginine methyl ester
MA	Mitochondrial anchorage
MGMT	O^6-methylguanine-DNA methyltransferase
Min	Multiple intestinal neoplasia
MLH1	MutL homologue 1
mM	Millimolar
MNU	Methylnitrosourea
MSH2	MutS homologue 2
MSI	Microsatellite instability
ncNOS	Neuronal constitutive nitric oxide synthase
NES	Nuclear export signal
NO	Nitric oxide
NO_2	Nitrite
NO_3	Nitrate
NSAIDs	Non-steroidal anti-inflammatory drugs
PBIT	S,S′-1,4-phenylene-*bis*(1,2-ethanediyl)*bis*-isothiourea
PBS	Phosphate-buffered saline
PG	Prostaglandin
PhIP	2-Amino-1-methyl-6-phenylimidazo[4, 5-b]pyridine
PI3K	Phosphatidylinositol-3′-kinase
PMS	Post Meiotic Segregation
PP2A	Protein phosphatise 2A
PGG_2	Prostaglandin G_2
PGH_2	Prostaglandin H_2
PVDF	Polyvinylidene difluoride

RB	Retinoblastoma protein
SC-51	L-N(6)-1-iminoethyl)lysine tetrazolamide
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulphate – polyacrylamide gel electrophoresis
Tcf/lef	T cell factor/lymphoid enhancer factor
TEAC	Trolox equivalent anti-oxidant capacity
TPA	12-*O*-tetradecanoylphorbol-13-acetate
Trypsin-EDTA	Trypsin-ethylenediaminetetraacetic acid
UV	Ultraviolet

1.0 INTRODUCTION

Alimentary tract diseases are major causes of morbidity and mortality all around the world. The digestive tract is particularly vulnerable to cancers, the incidence of which shows significant geographical variation (Johnson, 2007). World-wide, colorectal cancer is the fourth most common cancer in men and third most common cancer in women (Parkin *et al*., 2005). Significant international variations in the incidence of colorectal cancer have been observed (Parkin, 2004; Center *et al*., 2009b). Colorectal cancer is one of the three most common causes of death in industrialised western countries, but it is significantly lower in less developed world. A diet low in fiber, obesity, physical inactivity, and smoking have been associated with high incidence of colorectal cancer in the developed world (Giovannucci, 2002; Giovannucci and Wu, 2006; Botteri *et al*., 2008). However, the incidence of colorectal cancer is on the rise in newly-developed countries (Center *et al*., 2009b), where at one time the risk was considerably lower (Parkin *et al*., 2005). The high rates of cancers observed in countries previously with low colorectal cancer incidences is attributed to Westernization of diet, increase in obesity, and inactivity (Center *et al*., 2009a). In Canada, colorectal cancer is the second leading cause of death from cancer in men and the third leading cause of death from cancer in women (Loraine *et al*., 2009).

Several factors contribute to the development of colorectal cancer. There is growing evidence that genetic predisposition, diet, and lifestyle habits are some of the major contributing factors for colorectal cancer development. Within populations at high risk of colon cancer development, the lifetime risk of developing the disease is approximately 5 - 6% (Johnson, 2007). Genetic syndromes such as familial adenomatous

polyposis (FAP), Peutz Jeghers syndrome, juvenile polyposis, and hereditary nonpolyposis colorectal cancer account for approximately 3% of all cases (Aaltonen *et al*., 1998; Salovaara *et al*., 2000; Percesepe *et al*., 2001; Samowitz *et al*., 2001) and are not responsible for the two-fold increased risk in first-degree relatives of sporadic colorectal cancer patients (de Jong *et al*., 2002). These observations suggest that low-penetrance genes are involved in the disease process, and the development of colorectal cancer is a combination of environmental and genetic factors (de Jong *et al*., 2002; Heavey *et al*., 2004).

Diet is one of the major environmental factors affecting cancer development. Among all the diet-determined cancers, colorectal cancer is one of the most strongly related to one or more components in diet (Wirfält *et al*., 2009). Consumption of diets rich in fiber, folate, and calcium have been associated with reduced risk of colorectal cancer (Giovannuci *et al*., 2002; Bingham *et al*., 2003; Norat and Riboli, 2003; Larsson *et al*., 2006), while diets rich in meat and fat increase the risk of colorectal cancer (Giovannuci *et al*., 1992; Norat *et al*., 2005). It has been suggested that inhibitory effects of dietary fiber on the development of colon cancer depend on the source of fiber (Maziya-Dixon *et al*., 1994; Kritchevsky *et al*., 1999). Mice fed with wheat bran fiber led to 40% lower incidence of dimethylhydrazine (DHM)-induced colon cancer as compared with mice fed with cellulose-containing diets (Kritchevsky *et al*., 1999), suggesting a role for other compounds besides fiber in prevention of colon cancer. A subsequent study reported that the orthophenolic content of wheat grain has the ability to prevent colon cancer cell growth in Caco-2 cell lines (Drankhan *et al*., 2003).

Collectively, these data suggest that wheat bran components confer a protective role in prevention of colon cancer cell growth.

Wheat (*Triticum aestivum* L.) is one of the three important cereal grains with a global production of about 600 million tonnes annually (Shewry, 2009). Wheat grain, a rich source of carbohydrates and protein, accounts for nearly 30% of grain consumption. Due to its unique properties, it is a major source of calories in human and animal diets. Wheat grains contain significant amounts of phenolic acids, mostly localized in the bran (Verma *et al*., 2008; Verma *et al*., 2009).

Plant phenolic acids are defined as bioactive non-nutrient compounds in fruits, vegetables, grains, and other plant-based foods (Liu *et al*., 2004). Phenolic acids play a major role in providing essential functions in the reproduction and growth of the plants and provide structural integrity to the cell wall (Klepacka and Fornal, 2006). Structurally, phenolic acid can be subdivided into benzoic acid and cinnamic acid derivatives (Kim *et al*., 2006). For example, vanillic and salicylic acids are benzoic acid derivatives whereas ferulic and caffeic acids are cinnamic acid derivatives (Abdel-Aal *et al*., 2001). A preliminary animal study demonstrated that mice that fed on all wheat-based diets gained 30% less weight as compared to mice on a regular diet and, in addition, mice fed with a diet made with CDC Teal wheat had lower number of azoxymethane (AOM)-induced aberrant crypt foci as compared to mice fed with rat chow diet (Chibbar *et al*., unpublished).

More recently a detailed study of wheat bran from six cultivars representing six market classes of wheat, identified eleven phenolic acids and a lignan which differed in their anti-oxidant capacities (Verma *et al*., 2009). Gallic acid showed the strongest anti-

oxidant activity followed by ellagic, caffeic, and chlorogenic acids (Verma *et al*., 2009). Phenolic acids can also confer their protective role in health by chelating metal ions (Liyana-Pathirana and Shahidi, 2006), detoxifying enzymes (Yoshioka *et al*., 1995), and inhibiting transcription factors that initiate and promote tumor growth (Yoshioka *et al*., 1995). However, the precise mechanism of cereal grains in colorectal prevention is not well understood.

To understand the mechanism of wheat bran in colorectal cancer prevention, we examined the effects of individual phenolic acids present in wheat bran on the growth of two human colon cancer cell lines (HT-29 and HCT 116). In addition, we studied the effects of individual phenolic acids on the expression of selected genetic markers in treated cells by immunohistochemical studies.

2.0 LITERATURE REVIEWS

2.1 Functions of the Colon

Under normal circumstances, the colonic epithelium is constantly renewed by crypt proliferative cells which migrate upward along the crypt-villi axis as they terminally differentiate (Lipkin, 1973). The average time for these cells to proliferate is one day, and approximately 25% of the colonic epithelium is rejuvenated everyday. Moreover, the crypt epithelial cells of the entire colon and rectum are replaced every three to four days (Lipkin, 1985). The cell renewal rate in the colon and rectum may be regulated neurally or hormonally. Mitotic rate in the rat colonic crypts is low following chemical sympathectomy and is increased by the treatment with Metaraminol, a drug that releases the norepinephrine from nerve terminals (Tutton and Barkla, 1980). Hormones and paracrines secreted by intestinal enteroendocrine cells and pancreatic cells promote epithelial and stem cell turnover (Medh and Thompson, 2000). Over expression of growth hormone including peptide-2 (GLP-2) and insulin-like growth factor (IGF) stimulate intestinal cell proliferation in mice (Ulshen *et al*., 1993; Tsai *et al*., 1997), of which GLP-2 prevents enterocytes apoptosis (Tsai *et al*., 1997).

The other factors which are significant in maintaining normal colonic mucosa are B-cell lymphoma 2 (Bcl-2) and Bcl-2-associated X (Bax). The colonic epithelial cells express the pro-apoptotic Bax protein while the colonic stem cells are rich in anti-apoptotic Bcl-2 protein (Krajewski *et al*., 1994; Jones and Gores, 1997). Therefore the proliferation rate is higher in the base of crypts, whereas apoptosis occurs mainly at luminal surface of human colonic epithelium. This role of Bcl-2 is further supported by

the finding that incidence of spontaneous and induced apoptosis is increased in the base of colonic crypts in Bcl-2 knock out C57BL/6 mice (Merritt *et al*., 1995).

2.2 Understanding Colorectal Cancer

Colorectal cancer is one of the most common malignancies in developed nations. It is the second leading cause of cancer deaths in Canada. In 2009, approximately 22,000 Canadians were diagnosed with colorectal cancer and there were 9,100 deaths. Incidence of colorectal cancer is higher in developed countries such as North America, Australia, and New Zealand, in which the population is more economically privileged and consumes a diet rich in animal-based food products (Rupnarain *et al*., 2004).

Most of the colorectal cancers are in either sporadic or familial form, based on two distinct genomic instability pathways: chromosomal instability (CIN) and microsatellite instability (MSI) (Yamamoto *et al*., 2002; Grady, 2004; Imai and Yamamoto, 2008). Tumors with CIN phenotype are characterized as aneuploid cancer with the suppressor pathway such as deregulation of deoxyribonucleic acid (DNA) replication check points and mitotic-spindle check points, whereas tumors with MSI phenotype are associated with the mutator pathway for diploid cancer (Grady, 2004; Imai and Yamamoto, 2008).

2.2.1 Sporadic Colorectal Cancer

Nearly 85% of sporadic colorectal cancer is found to have CIN phenotype and is characterized with aneuploid cells, multiple chromosomal rearrangements, and accumulation of somatic mutations in oncogenes such as K-ras as well as tumor suppressor genes (Imai and Yamamoto, 2008). Spontaneous somatic mutations in tumor suppressor adenomatous polyposis coli (APC) gene is an early event in colorectal

tumorigenesis and accounts for nearly 80% of sporadic colorectal cancer and in about 50% of sporadic adenomas (Miyoshi *et al*., 1992; Powell *et al*., 1992; Imai and Yamamoto, 2008). Thus, combining these data, spontaneous somatic mutation in colonocytes results in loss of wild type APC allele, thought to be associated with development of sporadic adenomatous polyps (Miyoshi *et al*., 1992).

Over 60% of all somatic mutations in APC occur within less than 10% of the coding sequence of the gene in a mutation cluster region in exon 15, between codons 1286 and 1513 (Miyoshi *et al*., 1992). Within this cluster, there are two hotspots for somatic mutations at codons 1309 and 1450 (Beroud and Soussi, 1996). APC mutations within the cluster region are usually nonsense mutations and result in a truncated APC protein that lacks all of the axin binding sites and one or two of its 20-amino acid β-catenin biding sites. Moreover, one of two promoter regions of APC, promoter 1A but not promoter 2A, has been shown to be hypermethylated in approximately 30% of colorectal cancer but not in adenomas (Hiltunen *et al*., 1997). Hypermethylation of promoter region 1A may impact second mutation and inactivation of APC leading to invasive colorectal cancer. Mutations within APC gene in sporadic colon cancer are identical to mutations of APC in familial adenomatous polyposis patients.

MSI due to mutation in mismatch repair genes occurs in approximately 15% of sporadic colorectal cancer (Imai and Yamamoto, 2008).

2.2.2 Inherited Colorectal Cancer Disorder

Epidemiological studies have suggested that at least 10 - 15% of colorectal cancer is dominantly inherited (Houlston *et al*., 1992). Two common types of inherited colonic

cancer include: the familial adenomatous polyposis (FAP) and the hereditary nonpolyposis colorectal cancer (HNPCC) (Lynch *et al*., 1966).

2.2.2.1 Familial Adenomatous Polyposis (FAP)

FAP is an autosomal dominant inherited disease accounting for about 1 to 3% of all colorectal cancer cases (Grady, 2003). FAP is characterized by development of hundreds to thousands of colorectal polyps in affected individuals. These multiple colonic adenomas usually develop during the preteen age in FAP patients, and continuously proliferate throughout the colon. Although large numbers of these benign tumors are not singly life-threatening, some of these benign tumors progress to become invasive lesions over a period of time. If left untreated, the risk for a FAP patient to progress a carcinoma is almost certain by 40 to 50 years of age.

FAP is caused by a germline mutation of an APC gene on chromosome 5q21 (Kinzler *et al*., 1991). APC is a tumor suppressor gene for colorectal cancer and produces a 312 kD protein consisting of 2834 amino acids (Polakis, 1997). APC is a multifunctional protein that contains of an oligomerization domain and an armadillo region in the N-terminus, a number of 15-amino acids and 20-amino acids repeats in its central portion, as well as a C-terminus that consists of a basic domain and binding sites for EB1 and the human disc large protein (Polakis, 1997; Fearnhead *et al*., 2001) (Figure 2.1).

The multiple domains of the APC protein allow it to interact with a wide range of cytoplasmic proteins such as glycogen synthase kinase-3β (GSK-3β), axin proteins, and β-catenin (Fearnhead *et al*., 2001; Senda *et al*., 2005). The oligomerization domain at the N-terminus of the APC protein allows APC to form homo-dimers with both wild type and

truncated mutant APC proteins (Polakis, 1997). The armadillo region of APC binds to the regulatory B56 subunit of protein phosphatase 2A (PP2A) (Seeling *et al*., 1999), an enzyme that binds to axin through its catalytic subunit (Hsu *et al*., 1999). PP2A may act as an antagonist to the GSK-3β phosphorylation of β-catenin (Seeling *et al*., 1999). The 15-amino acids and 20-amino acids repeat motifs provide binding sites for β-catenin after phosphorylation of each site by GSK-3β (Su *et al*., 1993; Munemitsu *et al*., 1995). The cytoplasmic APC protein has two nuclear localization signals (NLS), explaining the presence of APC both in nucleus and cytoplasm (Zhang *et al*., 2000a) (Figure 2.1). In addition, APC also has a nuclear export signal (NES), which is important for APC shuttling of β-catenin between nucleus and cytoplasm (Zhang *et al*., 2000a).

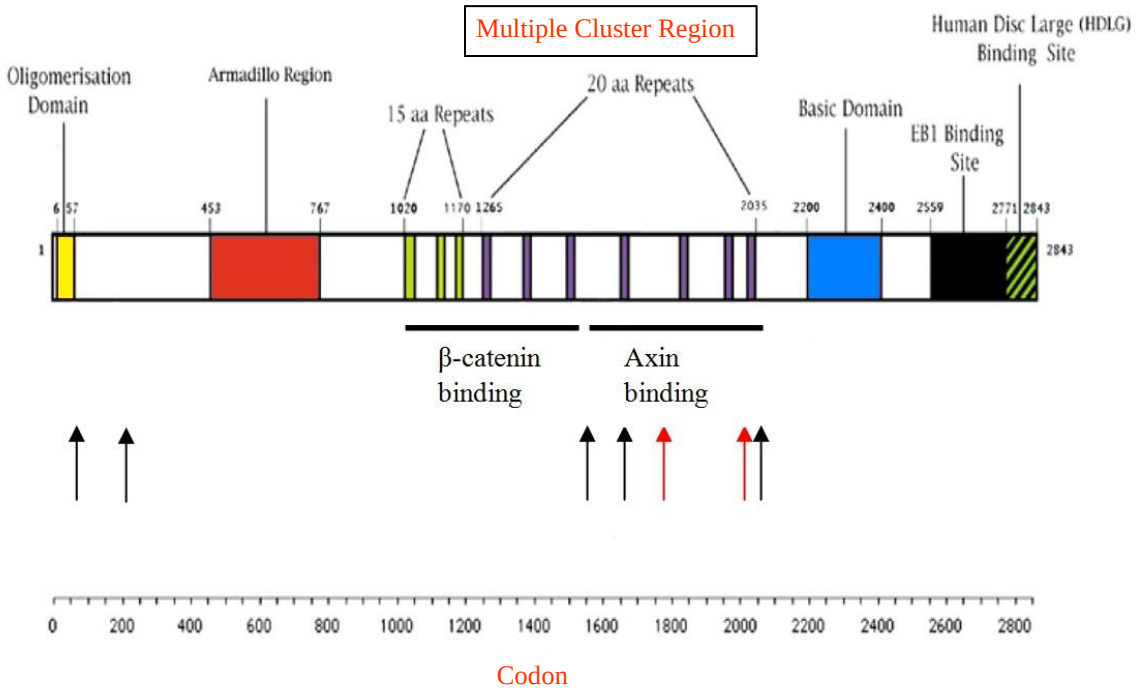


Figure 2.1. Functional domains of APC protein. APC is a large protein of 2843 amino acids, comprising several protein-interaction domains as summarized. Black arrows show active nuclear export signals (NESs). Red arrows indicate sites for nuclear localization signals (NLSs). (Adapted from Fearnhead *et al*., 2001)

A tumor suppressor gene loses its tumor-suppressing function when both alleles at homologous chromosomes are completely impaired. APC can produce normal gene product to suppress cellular proliferation as long as one of the allele is intact. Epidemiological studies on retinoblastoma show that mutation in the first allele is followed by inactivation of the second allele either through somatic APC mutation or loss of heterozygosity at this locus (Knudson, 2001). All individuals born with one mutant allele developed hundreds to thousands of adenomatous polyps in colon during their twenties and loss of both copies of APC gene causes the development of cancer (Behrens *et al*., 2005; Jaiswal *et al*., 2005).

More than 300 different mutations in APC gene have been reported to cause FAP (Laurent-Puig *et al*., 1998). Patients with FAP show an interesting genotype and phenotype correlation with respect to the position of inherited APC mutation. Germline mutations in APC are spread between codons 200 and 1600, with peaks at codons 1061 and 1309, and rarely go beyond codon 1600 (Beroud and Soussi, 1996). Most of the mutations, about 95%, are nonsense or frame shift mutations producing truncated APC protein (Beroud and Soussi, 1996; Laurent-Puig *et al*., 1998). As much as 60% of patients develop duodenal tumors in which these tumors are usually located at the ampulla or the periampulla region (Kashiwagi and Spigelman, 2000).

APC protein is an integral part of Wnt signal transduction pathway (Bienz and Clevers, 2000; Fearnhead *et al*., 2001), and plays an important role in cell-cell adhesion and apoptosis (Morin *et al*., 1996; Fearnhead *et al*., 2001). When Wnt binds to its receptor frizzled, GSK-3β is inhibited and results in β-catenin stabilization and accumulation in cytoplasm. The excess β-catenin translocates into the nucleus and binds

to the transcriptional activator *t cell factor/lymphoid enhancer factor* (tcf/lef), leading to transcription of target genes. When APC is present in a cell, it binds to β-catenin, axin, and GSK-3β to form a large protein complex, to facilitate phosphorylation of β-catenin by casein kinase 1 (CK1) and then GSK-3β, resulting in proteosome-dependent degradation of phosphorylated β-catenin (Moon *et al*., 2004; Senda *et al*., 2005) (Figure 2.2a).

The loss of functional APC protein or β-catenin prevents GSK-3β-mediated phosphorylation and subsequent β-catenin degradation, resulting in β-catenin accumulation (Munemitsu *et al*., 1995), which, in turns, translocates into nucleus and switch on transcription factors tcf/lef (Behrens *et al*., 2005) (Figure 2.2b). Targets for transcriptional activation by β-catenin include the oncogene c-myc (He *et al*., 1998) and Cyclin D1 (Shtutman *et al*., 1999; Tetsu and McCormick, 1999), which regulate cell cycle progression. The role of β-catenin in regulating c-myc and Cyclin D1 strongly suggests that loss of APC function deregulates genes and leads to increased cell proliferation in mucosa.

It has also been shown that APC participates in apoptosis regulation (Morin *et al*., 1996). Incorporation of a full length APC into HT-29 cells resulted in cell growth inhibition via increased apoptosis. In another study, APC was shown to regulate cyclooxygenase-2 (COX-2) (Araki *et al*., 2003). A wild type APC induced into HT-29 cells down regulated COX-2 expression. COX-2 promoter did specifically bind to Tcf-4 in an electrophoretic mobility shift assay (EMSA). Thus, COX-2 is one of the downstream target of the APC/Wnt signalling, in which COX-2 expression is down-regulated by APC and up-regulated by nuclear β-catenin.

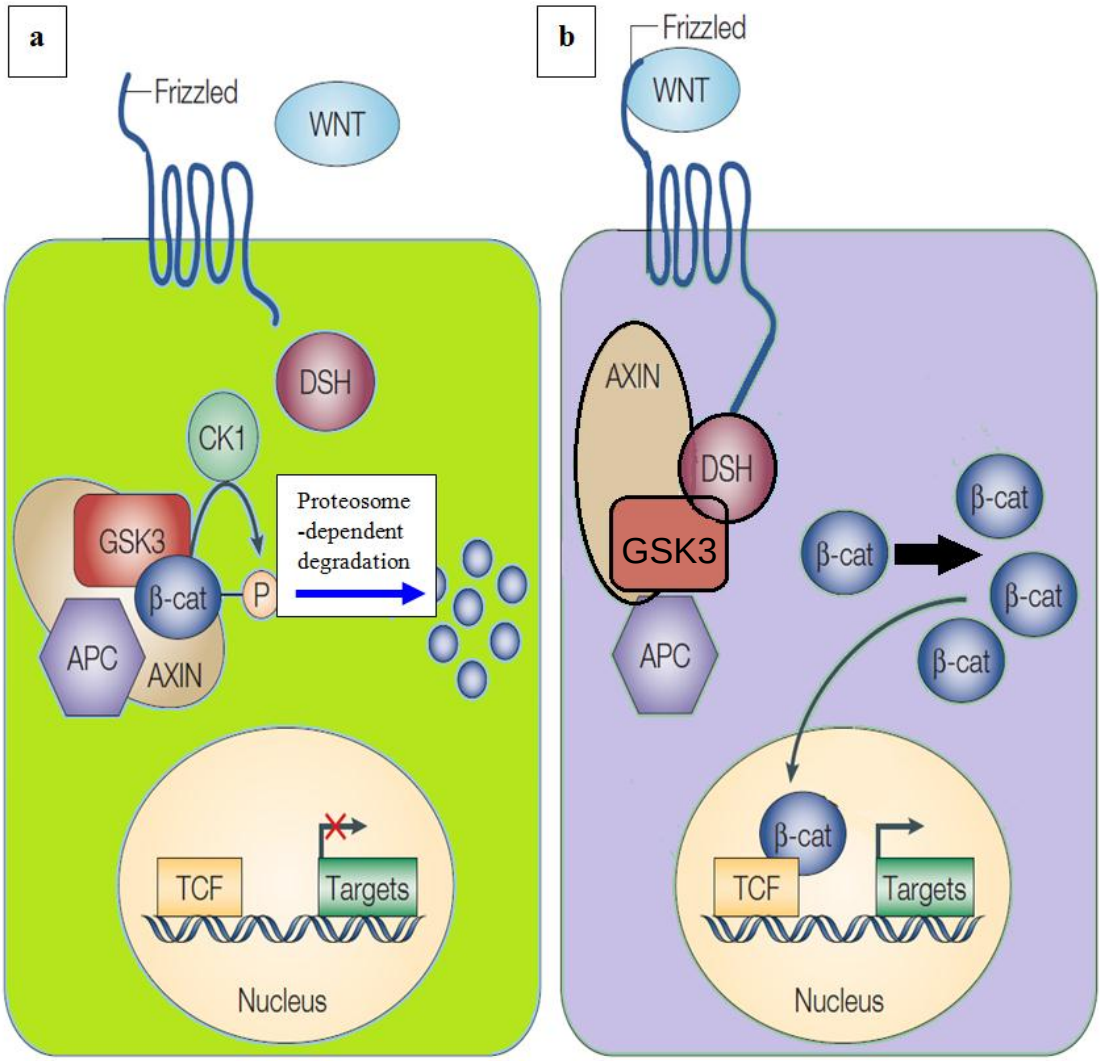


Figure 2.2. The Wnt signalling pathway. (a) In the absence of active Wnt, APC associates with β-catenin and axin, phosphorylates β-catenin by CK1 and then GSK-3β. The phosphorylated β-catenin undergoes proteasome-dependent degradation resulting in inhibition of Wnt signalling pathway. (b) The binding of Wnt to Frizzled receptor activates phosphoprotein Dishevelled (DSH) and inactivates GSK-3β, reducing phosphorylation and degradation of β-catenin. As β-catenin accumulates, it transports into the nucleus and binds to tcf/lef transcriptional activators, leading to the expression of target genes. (Adapted from Moon *et al*., 2004).

2.2.2.2 Hereditary Non-polyposis Colorectal Cancer

HNPCC is an autosomal dominant inherited disease which accounts for 3 to 5% of all colorectal cancer (Mecklin, 1987; Aaltonen *et al*., 1998). Individuals with HNPCC develop carcinomas at an average age of 44 years if left untreated (Lynch *et al*., 1993). Precursor adenomas show a villous histology feature.

Defective DNA repair caused by inactivation of DNA mismatch repair genes is both fundamental and initiating event in HNPCC. Most cases of HNPCC are linked to mutations in genes of bacterial *mutS* and *mutL* gene family which encode homologues of bacterial post replicative DNA mismatch repair proteins MutS and MutL (Kolodner, 1996). The most commonly found mutations in HNPCC are in human MSH2 (*mutS* homologue 2) and MLH1 (*mutL* homologue 1) (Umar *et al*., 1996).

As in APC-β-catenin scheme, there is accumulation of mutations in HNPCC but different genes are involved. There are dozens of mismatch repair proteins in human cell system which coordinate in recognizing and repairing the repetitive misalignment during DNA replication and thus maintain fidelity of DNA replication. The identified mismatch repair genes are listed in Table 1 (data modified from Lynch and de la Chapelle, 2003). DNA mismatch repair is initiated by two different complexes: a complex between MSH2-MSH6 for the recognition of single base mismatches and a complex between MSH2-MSH3 for the recognition of two to four base pair insertions or deletions (Kolodner, 1996; Umar *et al*., 1996). Subsequently, a complex between MLH1 and another *mutL* homologues, PMS1 or PMS2 (Post Meiotic Segregation) interacts with MSH2 that binds to a base mispair. The 3'-5' exonuclease (EXO1) or PNCA are

believed to interact with MSH2/MLH1 in excision, resynthesis, or ligation (Umar *et al*., 1996).

Mutations in mismatch repair genes cause alteration of microsatellites, leading to MSI. MSI, a condition of instability in microsatellite length is a hallmark of tumors in HNPCC (Peltomaki *et al*., 1993). Microsatellites are genomic regions made up of short DNA sequences or merely a single nucleotide, often 1 to 5 nucleotides. The increase or decrease in number of DNA repeats in a tumor compared to number of repeats in the same region in non-tumor from an individual is referred as "MSI." In normal cells, the misalignment is repaired by caretaker genes. Patients with HNPCC inherit one mutant DNA repair gene ("the first hit") and one normal allele (Knudson, 2001).

For unknown reasons, cells in some organs such as colon, stomach and endometrium are susceptible to a second, somatic mutation ("the second hit"), which inactivates the normal allele, causing loss of heterozygosity. The mutation rates are up to 1000 times higher than normal with homozygous loss of mismatch repair genes, and most HNCPP tumors show microsatellite instability. Most microsatellite sequences are in noncoding regions of genes. However, some microsatellite sequences are located in coding or promoter regions of genes involved in cell growth regulation. These genes include type II TGF-β receptor and Bax. TGF-β signaling inhibits growth of colonic epithelial cells, and Bax gene causes apoptosis (Grady, 2004). Loss of mismatch repair results in accumulation of mutations in these and other growth-regulating genes, culminating in the emergence of colorectal carcinomas.

However, lack of any of these proteins in tumors will be inefficient to recognize and correct replication errors, thus causing genetic instability. These proteins contain

different efficiencies for recognizing and repairing a wide range of base mispairings (Kramer *et al*., 1984). For instances, G:T and A:C base mispairings are very efficiently corrected, whereas the C:C base mispairing is the worst substrate for mismatch repair. These mutations, in some microsatellite regions, cause misalignment of repetitive subunits. Germline mutations in mismatch repair genes, predominantly MSH2 or MLH1, have been found to cause 90% of cases in patients with HNPCC (Cruz-Correa and Giardiello, 2002; Lynch and de la Chapelle, 2003) (Table 1.1). However, Lynch syndrome is less frequently caused by germline mutations in MSH6 and is rarely caused by PMS2 (Lynch and de la Chapelle, 2003; Abdel-Rahman *et al*., 2006) (Table 1.1).

Table 1.1. The identified mismatch repair genes which cooperated as site of mutation in HNPCC.

Genes	**Chromosomal location**	**Percentage of HNPCC mutations detected**
MLH1	3p21	50.9%
MSH2	2p16	37.6%
MSH6	2p16	9.6%
PMS1	2q31	0.3%
PMS2	7q11	1.6%

2.3 Pathogenesis of Colorectal Cancer

2.3.1 Adenoma-Carcinoma Pathogenesis of Colorectal Cancer

In the colorectal carcinoma, accumulation of genetic changes underlies development of neoplasia. In the early 90's, Vogelstein and Fearon (1990) hypothesized that colorectal cancer is a multi-step process (Figure 2.3) in which genes are mutated in a

specific order (Fearon and Vogelstein, 1990), resulting in the transition from normal mucosa to benign adenoma to severe dysplasia to frank carcinoma. The first in this process is loss of APC tumor suppressor gene which results in loss of deleted in colon cancer (DCC) gene and activation of K-ras oncogene (Figure 2.3). This is followed by inactivation of p53, leading to eventual carcinoma formation (Figure 2.3). This is in agreement with Knudson's two hit theory in which carcinogenesis process is thought to result from accumulation of two or more mutations that affect cell cycle control aberrations or other features of neoplastic development (Knudson, 2001).

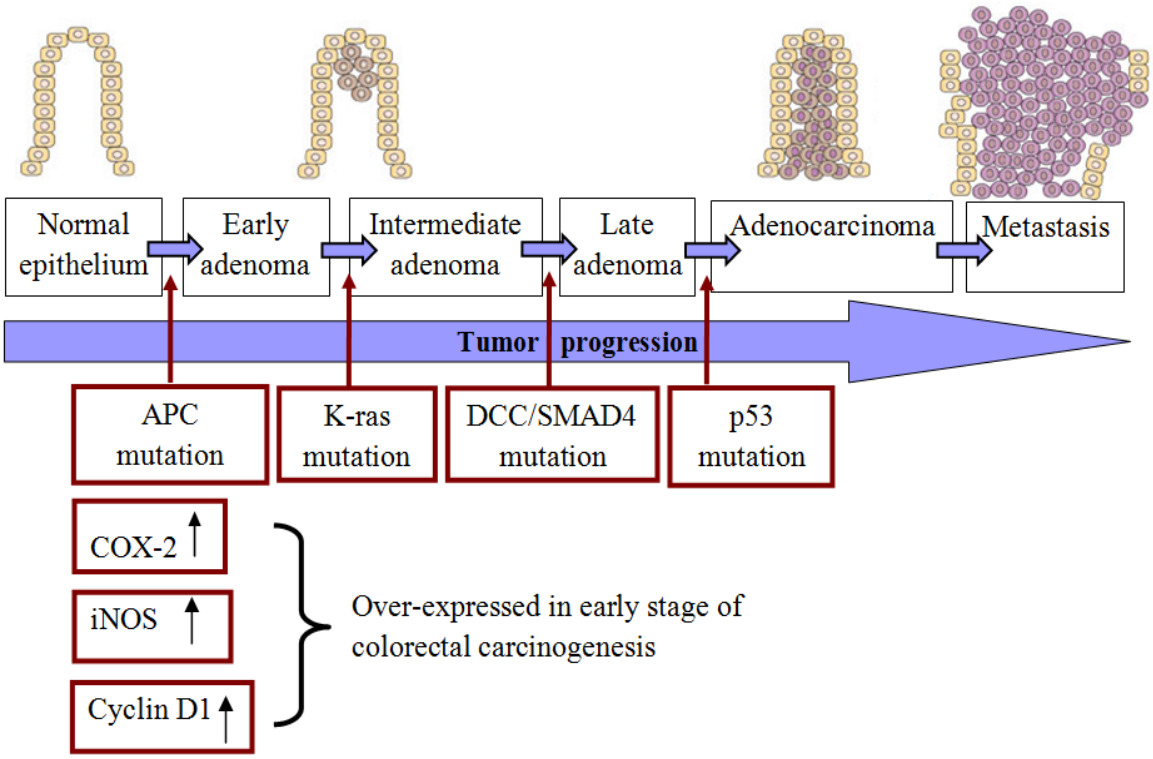


Figure 2.3. Colorectal cancer occurs in a multi-step process, in which four genes are mutated in a specific order. (Adapted from Baldassarre *et al*., 2004; Green and Hudson, 2005)

The inactivation or alteration of the APC/β-catenin in Wnt signaling pathway has wide spectrum of negative downstream effects with multiple cellular functions and interactions, including transcriptional regulation, chromosomal instability, cell migration, intracellular adhesion, and regulation of cell cycle, proliferation, differentiation, and programmed cell death (Behrens *et al*., 2005; Jaiswal *et al*., 2005). Over the decades, development of most colorectal cancers is believed to be initiated by inactivation of one APC allele, followed by inactivation of a second APC allele as discussed earlier (Knudson, 2001), usually through a mutation or deletion and epigenetic hypermethylation in CpG islands of APC promoter region (Hiltunen *et al*., 1997; Arnold *et al*., 2004).

DCC gene located on chromosome 18q21.2 (Fearon *et al*., 1990), encodes for dependence receptors for netrin-1 in colonic and other tissues, thus establishing its role as a tumor-suppressor gene in human cancer (Arakawa, 2004; Mazelin *et al*., 2004). DCC gene product bound to netrin-1 mediates signals for cell proliferation, differentiation, or migration (Mazelin *et al*., 2004). In the gut, netrin-1 plays a role in mucosal integrity, epithelial cell migration, and tissue renewal by inducing cell survival in proliferating crypt progenitors (Mazelin *et al*., 2004; Bredesen *et al*., 2005). The netrin-1 level is low at the villus tip therefore DCC promotes apoptosis and cell shedding because cell death receptors are expressed constitutively throughout crypt-villus axis. In patients with colorectal cancers associated with inflammatory bowel diseases, the anti-apoptotic ligand netrin-1 is upregulated causing colorectal cancer to progress (Paradisi *et al*., 2009). Therefore, loss or reduction of DCC expression is proposed to have an important role in the transition of an intermediate adenoma to an advanced adenoma (Vogelstein *et al*., 1988). Several reports have suggested that DCC inactivation is a late event in

carcinogenesis of colorectal cancer and has a role in metastasis (Itoh *et al*., 1993; Ookawa *et al*., 1993; Kato *et al*., 1996).

The loss of K-ras gene in RAS/RAF signaling pathway and p53 gene are believed to be late genetic events in colorectal pathogenesis (Jaiswal *et al*., 2005). Among the multiple genetic steps in human colorectal carcinogenesis, K-ras mutation is now widely considered to be a hallmark of colorectal cancer in humans. K-ras gene encodes for a guanine nucleotide binding protein transmitting signals from membrane-bound tyrosine kinases through a group of downstream regulators to the nucleus (Lowy and Willumsen, 1993). When a specific mutation occurs in the K-ras gene, the Ras protein is locked in its active state (Robbins and Itzkowitz, 2002), activating the signal pathway resulting in an unregulated proliferation of colonocytes and transition of adenomas to carcinoma. K-ras point mutations have been detected in about one-half of colon tumors (Forrester *et al*., 1987) and in about 60% of colonic aberrant crypt foci in humans (Losi *et al*., 1996). K-ras mutation has also been found in approximately 60% of colon tumors chemically induced in rats by DMH or AOM (Jacoby *et al*., 1991). Wu and coworkers (2010) reported that K-ras induced the growth of HT-29 colon cancer cell line through expressions of COX-2, EP_1 or EP_4, GSK3β and increased Tcf transcriptional factor.

The p53 has been described as "the guardian of the genome" because of its role in maintaining genome stability and integrity by preventing genomic mutations, hence it has become one of the most common targets to look for genetic alteration in human tumors (Liu and Gelmann, 2002). Mutations in p53 are believed to play an important role in the transition from advanced adenoma to frank carcinoma, with nearly 50% of high-grade adenomas and close to 75% of cancers exhibit p53 mutations (Robbins and Itzkowitz,

2002). The p53 tumor suppressor gene located on chromosome 17p13.1 coding for a DNA binding protein localized in the nucleus (Liu and Gelmann, 2002). The major functions of the p53 protein are cell cycle arrest and stimulation of apoptosis in response to DNA damage such as irradiation, ultraviolet (UV) light, or mutagenic chemicals (Liu and Gelmann, 2002). Activation of normal p53 in response to DNA damaging agents leads to cell cycle arrest in late G_1 phase and induction of DNA repair by transcriptional upregulation of cyclin-dependent kinase inhibitor p21 and the GADD45 genes. Cells proceed with cell cycle if the damaged DNA is repaired successfully; if not, cells with mutated DNA are destroyed by induction of apoptosis-inducing genes such as Bax. In cells with homozygous loss of p53, DNA damage is not repaired and accumulates the mutation and causes malignant transformation (Liu and Gelmann, 2002).

2.4 Mouse Models for Colorectal Cancer

Rodent and human colons share many biological functions, histological and genetic features (Corpet and Pierre, 2005). Therefore rodent models have been used to study mechanisms of carcinogenesis, identification of novel target genes and biomarkers, and screen potential chemopreventive agents and their mechanisms of chemoprevention. A recent systematic review and meta-analysis of colon chemoprevention in 6,714 human volunteers, 3,911 rats, and 458 mice, showed that differences between models was small and that rodent models roughly predicted effects in humans, but the prediction is not accurate for all agents (Corpet and Pierre, 2005). These analyses showed that carcinogen-induced rat studies matched human trials for aspirin, calcium, carotene, and wheat bran whereas Min mice results were comparable with human results for aspirin.

Since the environmental factors such as diet strongly influence progression of colorectal cancer, efforts were made to generate mice that carry gene mutations implicated in progression or initiation of colorectal cancer to study gene-environment interactions. In addition, it is necessary to use carcinogens such as DMH or AOM due to extremely low spontaneous rates of colon cancer in laboratory rodents (Corpet and Pierre, 2005). Tumors in AOM-induced rats share many histopathologic features with human tumors, and in a manner similar to ACF, adenoma, and carcinoma sequences (Takahashi *et al*., 2004). Colonic tumors induced by DMH or AOM show close similarity in histological features and proliferation characteristics to human colonic neoplasms (Hamilton *et al*., 1982). Therefore, rats exposed to DMH or AOM induced colonic tumors are considered a good model to simulate colon carcinogenesis in humans. Additionally, the heterocyclic amines such as 2-amino-1-methyl-6-phenylimidazo [4, 5-b] pyridine (PhIP) have been used to induce tumors in rats and mice through the induction of APC and β-catenin mutations in the colon (Tsukamoto *et al*., 2000). These carcinogen-induced tumors in rodents show an implication of Wnt/β-catenin/Tcf pathway during progression of colorectal cancer and over-expression of COX-2 and inducible nitric oxide synthase (iNOS) in these tumors (Corpet and Pierre, 2005).

2.4.1 Mouse Models for Colorectal Cancer and FAP

The first mouse that had a mutation in the APC gene was termed multiple intestinal neoplasia (APC^{Min}) due to its development of more than 100 adenomas in small intestine (Su *et al*., 1992). These mice were found to have nonsense mutation in the APC gene at codon 850 resulting in a truncated protein of 850 amino acids. APC^{Min} heterozygotes are born normally but have on average life span of four to six months.

Similarly to the APC^{Min} mice, Oshima and coworkers (1995) generated a mouse designated $APC^{\Delta 716}$ which develop multiple adenomas throughout the gastrointestinal tract especially in the small intestine, and have a reduced life span. The use of conditional APC allele induces tumors in rat colon instead of small intestine (Shibata *et al*., 1997). The deletion of a conditional allele APC^{580S} allele leads to development of multiple colonic adenomas within four weeks.

2.4.2 Mouse Models for HNPCC

MSH2 was one of the first HNPCC genes to be mutated in mice (Reitmair *et al*., 1995). These mice developed gastrointestinal and skin adenomas and carcinomas. Fifty percent of the MSH2 homozygous mutant mice die by the age of six months and all mice die by the age of 12 months. These mice died early in their life, due to T cell lymphomas containing microsatellite instability (Lowsky *et al*., 1997). Mice with MSH6 deficiency developed invasive B and T cell lymphomas and tumors in gastrointestinal tract; however, they did not show microsatellite instability (Edelmann *et al*., 1997). Fifty percent of the MSH6-deficient mice died by 10 months of age and all of the mice died by the 18 months of age. Therefore, MSH6 homozygous mutant mice have longer life spans than that of the MSH2 homozygous mutant mice.

The inactivation of MLH1 in mice causes microsatellite instability, infertility, and tumor susceptibility (Baker *et al*., 1996; Edelmann *et al*., 1996). MLH-/- mice do not produce mature sperm due to prematurely separated chromosomes and arrest in the first division of meiosis prior to entering the apoptotic pathway. MLH1-deficient female mice do not produce fertile eggs. Fifty percent of MLH1-deficient mice died by the age of six months and 100% died by 13 months of age, due to the development of T cell

lymphomas, gastrointestinal adenomas and early invasive carcinomas, especially in the small intestine.

In PMS2 deficient mice, the females have normal fertility but not the males, due to abnormalities in chromosome synapsis and non homologous pairing during prophase of meiosis I (Baker *et al*., 1995). PMS-/- mice are prone to have microsatellite instability and predisposition to sacromas and lymphomas; however, these mice do not develop tumor in their gastrointestinal tract.

2.5 Chemoprevention of Colorectal Cancer by Dietary Fiber – Phenolic Acids

2.5.1 Dietary fiber (DF)

Dietary fiber is predominantly found in plant-based foods such as vegetables, grains, pulses, nuts and fruits (Anderson *et al*., 2009). According to Trowell's original definition, dietary fiber is "that portion of food which is derived from the cellular walls of plants which is digested very poorly by human beings" (Trowell, 1972). Over the years, the definition of dietary fiber has been discussed at length, to precisely define DF constituents. The American Association of Cereal Chemists (AACC 2001) defined DF as "the edible parts of plants or analogus carbohydrates that are resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine. That includes polysaccharides, oligosaccharides, lignin, and associated plant substances." The European Commission (2008) has further refined the DF constituents to include edible carbohydrate polymers with three or more monomeric units, which are neither digested nor absorbed in the human small intestine. These polymers could be obtained from raw or processed food or be synthetic.

However, the Codex Alimentarius Committee on Nutrition and Foods for Special Dietary Uses (CCNFSDU), the World Health Organization, and the Food and Agriculture Organization agreed that carbohydrate polymers with ten or more monomeric units should be included in DF (CNFSDU-WHO/FAO Codex Alimentarius Commission, 2008). A simplistic DF definition is "any dietary component that reaches, without being absorbed, in a healthy human gut" (Ha *et al*., 2000). The total dietary fiber (TDF) includes soluble dietary fiber (SDF) and insoluble dietary fiber (IDF) based on their solubility in water (Asp *et al*., 1983). In humans, DF influences human health through several mechanisms. For instance, SDF first acts in the stomach by slowing the transit time and reducing the nutrient absorption and the remainder is fermented by microbiota in the proximal colon (James *et al*., 2003).

2.5.2 The Role of Dietary Fiber in Colorectal Cancer

The role of dietary fiber in prevention of colon carcinogenesis was first suggested by Burkitt (Burkitt, 1970; Burkitt, 1971). He showed an inverse relationship between dietary fiber intake and the risk of colorectal cancer incidence after he observed that black Africans had much lower incidence of colon cancer than did Caucasian Africans. The former population's diet was composed mainly of grains and unrefined carbohydrate foods while the latter population adhered to Western diet which focused on animal protein and contained low amounts of plant-based food.

Since then, dietary fiber intake has held the interest of scientists due to the possibility of being a major factor influencing onset of colon cancer. The role of DF to prevent colon cancer was further reinforced when lower incidence of colon cancer was observed in Finnish residents as compared to those in New York (Reddy *et al*., 1978).

Both the populations consumed a high-fat diet, but the Finish group also consumed a high-fiber diet. Over the last three decades, a large body of epidemiological and animal studies have proposed that intake of food high in dietary fiber can reduce the incidence of colorectal cancer. Several animal studies indicate a protective effect of dietary fiber. For example, addition of 15% pectin to a semi-purified diet containing 20% fat, inhibited AOM-induced tumors in Fischer 344 rats, but it had no corresponding effect on the methylnitrosourea (MNU)-induced tumors in rats (Watanabe *et al*., 1979). The researchers inferred that pectin interferes with the metabolic activation of AOM in the colon, which is necessary for carcinogenesis. On the other hand, the addition of 15% wheat bran inhibited both AOM- and MNU-induced colon tumors in Fischer 344 rats. In another study, the authors reported that diets containing 35% beef fat and 10% wheat bran, alfalfa, or cellulose did not inhibit AOM-induced colon tumors. Diets with 5% fat and 20% or 30% wheat bran or cellulose reduced AOM-induced tumors (Nigro *et al*., 1979). Recently, an epidemiological study showed that daily consumption of 48g of whole grain wheat breakfast cereal for three weeks has pronounced a prebiotic effect on human gut microbiota (Costabile *et al*., 2008). These findings suggest that the type of fiber is important in modulating the effects of a colon carcinogen and wheat bran has the most consistent inhibiting effect.

2.5.3 Possible Dietary Fiber-mediated mechanisms in prevention of colon cancer

Although the mechanisms by which DF reduces the incidence of colon cancer is uncertain, it is now widely known that digestion of fibers has several physiological effects on the intestinal and colon luminal environment. The protective effects of DF may be mediated by its physical form and its interaction with other dietary components

(Kritchevsky *et al*., 1997). DF ingestion increases intestinal transit time, which would permit less contact time between potential carcinogens in the lumen and in the gut mucosa (Burkitt, 1970; Cummings, 1984; Spiller *et al*., 2003; Vuksan *et al*., 2008).

DF also improves concentrations of anti-carcinogenic short-chain fatty acids, and augments stool bulk directly, thus diluting potential carcinogens in the large intestine (Fleming *et al*., 1983; Topping and Clifton, 2001; Hawk *et al*., 2005; Vuksan *et al*., 2008). Bile acids are less soluble at lower pH and therefore there is less conversion of primary bile acids to toxic secondary bile acids. The acidification of the colonic contents promote the binding of calcium to free bile acids, thus prohibiting their effects on colonic cells (Wargovich *et al*., 1984). The increased colonic acidification inhibits colonic bacterial enzyme 7α-dehydroxylase which degrades primary bile acids to secondary bile acids (Thornton, 1981).

The possible protective effects of wheat bran include dilution of colonic contents (bile acids, long chain fatty acid, and carcinogens), acceleration of colonic transit, alteration of energy metabolism by a fermentation product, butyric acid, and promotion of microbial growth (Kritchevsky *et al*., 1997; Lupton and Turner, 1999). In an animal study with Sprague-Dawley, rats fed with a wheat bran diet showed the best dilution ability in cecum, proximal, and distal colon. In the same study, wheat bran was reported as the best diluter, followed by cellulose, oat bran, guar gum, pectin, and fiber free diet (Gazzaniga and Lupton, 1987). In another *in vivo* study with Sprague-Dawley rats, wheat bran was shown as the best accelerator of colonic transit, followed by oat bran, pectin, guar gum, cellulose, compared to fiber free diet which showed the longest transit time (Lupton and Meacher, 1988). Fiber is metabolized by intestinal microflora with the

production of short-chain fatty acids, such as acetate, propionate and butyrate (Scheppach *et al*, 1995; Kritchevsky *et al*., 1997). All of the short-chain fatty acids can stimulate colonic epithelial proliferation; however, butyrate also has the ability to cause histone acetylation, apoptosis induction, differentiation induction, and regulate expression of some oncogenes (Scheppach *et al*., 1995).

Epidemiological studies have shown a direct relationship between fecal pH and colon cancer incidence in human populations (Bruce, 1987). Hypothetically, the degradation of normal fecal components to potential carcinogens by bacteria may be inhibited by increased fecal acidification (Thornton, 1981; Harris and Ferguson, 1993).

Fiber fermentation by bacteria increases colonic and fecal short chain fatty acid production. Short chain fatty acids, primarily acetate, propionate, and butyrate, are organic acids produced within the intestinal lumen by bacterial fermentation of predominantly undigested dietary carbohydrates; however, these organic acids are also produced in a minor part by dietary and endogeneous proteins such as mucus and sloughed epithelial cells (Topping and Clifton, 2001). Carbohydrate is preferred over protein as a substrate for fermentation by most microbes. Carbohydrate fermentation by saccharolytic bacteria occurs mainly in the proximal colon whereas fermentation of proteins and amino acids by proteolytic bacteria usually takes place in the distal colon where fermentable carbohydrates and water have been depleted (Macfarlane *et al*., 1992; Topping and Clifton, 2001). Compared to proximal, the distal part of the colon is considered less favorable for microbes due to the production of toxic metabolites such as ammonia, sulphur-containing compounds, indoles, and phenols as well as the higher pH (Topping and Clifton, 2001). These are some of the reasons which have been suggested

for distal colon as the prevalent location of several gastrointestinal disorders such as ulcerative colitis and colon cancer (Marteau, 2006; Le Leu *et al*., 2006).

The production of short-chain fatty acids also serves as a primary energy source for normal colonic epithelium, which contributes to about 5 to 15% of human total caloric requirement and stimulates growth of colonic epithelium (Bergman, 1990). Among the short-chain fatty acids, butyrate is the preferred fuel for colonic epithelial cells where 70% to 90% of butyrate is metabolized by colonocytes (Cook and Sellin, 1998). Butyrate blocks cell cycle at G1 by inducing $p21^{WAF1/CIP1}$ protein which inhibits cell proliferation (Archer *et al*., 1998). This blockage of cell cycle at G1 might allow DNA checkpoint-mediated repair of genomic instability or mutations (Scheppach and Weiler, 2004). Fiber associated with high butyrate concentrations was shown to reduce tumor mass in 1, 2-DMH induced tumorigenesis in Sprague-Dawley rats (McIntyre *et al*., 1993). In another study, short-chain fatty acids such as butyrate induced mitochondrial pathways of apoptosis by up-regulating Bcl-2 homologous antagonist killer (Bak) and Bax while downregulating Bcl-2 and B-cell lymphoma-extra large (Bcl-xL) in Caco-2 colon cancer cells (Ruemmele *et al*., 2003).

Theoretically, the degree of the fiber source protective against colorectal cancer development is based on its fermentation ability, which provides higher short-chain fatty acid concentrations to maintain the normal colonic cell proliferation and differentiation. Besides dietary fiber, phenolic acids have also been shown to prevent colon cancer.

2.5.4 Phenolic Acids

Almost all plants and most of the plant-derived foods contain numerous and significant amounts of phenolic compounds, a group of natural products, which includes

monomeric cinnamic acids (Herrmann, 1989). Higher concentrations of these natural products are commonly found in cereal bran, the outer protective layers of a cereal grain (Baublis *et al*., 2002). For example, ferulic acid was found to be one of the major phenolic acids in cereal grains, especially in wheat bran (Verma *et al*., 2009). Most of these phenolic compounds are derivatives of amino acids, phenylalanine or tyrosine, followed by deamination and introduction of one or more hydroxyl substituents into the phenyl ring to form phenolic acids (Pereira *et al*., 2009). There are two major categories of phenolic acids in cereal grains: benzoic acid or cinnamic acid derivatives. These compounds are derived from a common carbon skeleton building block: the C_6-C_1 benzoic acids and the C_6-C_3 cinnamic acids (Rice-Evans *et al*., 1996; Pereira *et al*., 2009). Another difference between the benzoic and cinnamic acid skeleton is the extension of the conjugated side chain due to the presence of an additional conjugated double bond in cinnamic acid structure. Benzoic acid is formed by a benzene ring with carboxyl (–COOH) side chain whereas cinnamic acid has a propenoic (–CH=CH–COOH) side chain attached to the benzene ring (Natella *et al*., 1999). For example, ellagic and vanillic acids belong to benzoic acid derivative phenolic acids while caffeic and ferulic acids belong to cinnamic acid derivatives phenolic acids. The difference in number and position of the hydroxyl groups on the aromatic ring contribute to the diversity in structure of phenolic acids. The gallic acid (benzoic acid derivative), has 3 hydroxyl groups on the 3, 4, and 5 position on the aromatic ring, whereas the ferulic acid (cinnamic acid derivative) has only one hydroxyl substitution on the 4 position on the aromatic ring.

2.5.5 Wheat – A Major Source of Energy in Human Nutrition

Worldwide, wheat is one of the popular cereal grains and a good source of dietary fibers (Alabaster *et al*., 1997). Phenolic acids are predominantly present in the outer protective layer of wheat, the bran layer (Baublis *et al*., 2002; Gallardo *et al*., 2006; Verma *et al*., 2009). The majority of phenolic acids found in a wheat grain exists as insoluble and bound forms such as esters, glycosides, and are often conjugated with organic acids (Liyana-Pathirana and Shahidi, 2006), whereas a small percentage of phenolic acids exists in soluble form (Stalikas, 2007). Wheat bran extracts exhibit greater anti-oxidant capacity than any other wheat grain fractions (Onyeneho and Hettiarachchy, 1992) and the ability to inhibit lipid oxidation catalyzed through peroxyl radicals (Baublis *et al*., 2000). More recently, Verma *et al*. (2009) showed the presence of eleven phenolic acids in wheat bran extracts from six Canadian wheat cultivars. The extracted phenolic acids also differed greatly in their anti-oxidant activities.

2.5.6 Role of Phenolic Acids in Chemoprevention

Naturally-occurring phenolic acids have been found to be strong anti-oxidants against free radicals and/or other reactive oxygen and reactive nitrogen species, which often play a role in cancer and cardiovascular diseases (Yu *et al*., 2002). The hydroxyl derivatives of both benzoic and cinnamic acids act as free radical scavengers, as they form a corresponding phenoxyl radical by donating a proton to the radical and terminating the radical chain reactions, which breaks the cycle of generation of new radicals (Pereira *et al*., 2009). The new radical form of the anti-oxidant has a much greater chemical stability then the initial radical, and thus these relatively long-lived

radicals are able to modify the free radical-mediated oxidation processes (Parr and Bolwell, 2002).

Moreover, phenolic acids are also able to chelate metal ions involved in the formation of free radical confers, thus conferring stronger anti-oxidant capacity to phenolic acids (Yang *et al*., 2001; Liyana-Pathirana and Shahidi, 2006). The hydrophobic property of the benzene rings and the hydrogen bonding potential of the hydroxyl substituent in phenolic acids, allows them to interact with proteins, and therefore act as anti-oxidants, by inhibiting the activities of enzymes involved in free radical generation (Shahidi and Wanasundara, 1992; Parr and Bolwell, 2002). Besides anti-oxidant activity, Yoshioka and coworkers (1995) reported that one of the possible anti-cancer mechanisms of phenolic acids was mediated through inhibiting AP-1, a transcription factor that initiates and promotes tumor growth.

Phenolic acids have been shown to prevent cancer induction in several experimental systems. In strain-A mice, gallic acid greatly inhibited lung tumors induced by chronic treatment with morpholine and sodium nitrite in drinking water (Mirvish *et al*., 1975). However, gallic acid had a slight inhibitory or no effect on tumorigenesis induced by nitrosomorpholine or mono-nitrosopiperazine. It was suggested that gallic acid could be useful as a preventative agent when co-administered with drugs that are readily nitrosatable. The phenolic acids of apples have been linked with prevention of colon cancer *in vitro* (McCann *et al*., 2007; Eberhardt *et al*., 2000). Apple extracts inhibited the growth of Caco-2 colon cancer cells in a dose-dependent manner (Eberhardt *et al*., 2000).

In HT-29, HT 115, and Caco-2 colon carcinoma cell lines, crude apple extract rich in phenolic acids, protected against DNA damage, improved barrier function, and inhibited invasion, key stages of carcinogenesis (McCann *et al*., 2007). The phenethyl ester of caffeic acid was evaluated in the C57B1/6J-Min/+(min/+) mouse model that has a germline mutation in the APC gene and is subject to spontaneous intestinal adenomas (Mahmoud *et al*., 2000). Addition of caffeic acid in the diet reduced tumor formation by 63% and decreased expression of β-catenin (Mahmoud *et al*., 2000). Gallic acid treatment in a concentration and time-dependent manner, inhibited the growth of human stomach (KATO III) and colon (COLO 205) cancer cell lines through the induction of apoptosis (Yoshioka *et al*., 2000). This suggested that gallic acid might be useful as an adjunct drug for treating digestive tract cancers that are resistant to traditional chemotherapeutic agents. Furthermore, wheat grain orthophenols were shown to prevent colon cancer cell growth in Caco-2 cell lines (Drankhan *et al*., 2003). In summary, the above observations suggest that phenolic acids can be natural alternatives to prevent various cancers, especially colon cancer, are the focus of this study.

2.6 Cyclooxygenase-2 (COX-2)

2.6.1 Cyclooxygenases (COXs)

COXs are rate-limiting enzymes in biosynthesis of biological mediators, prostaglandins (PGs), which have a variety of physiological effects such as regulation of cell proliferation, cell motility, inflammatory mediation, and apoptosis (Sheng *et al*., 1998; Stolina *et al*., 2000; Sheng *et al*., 2001). COX-1 gene is mapped to chromosome regions 9q32-q33.3 and COX-2 is located on 1q25.2-q25.5, encoding the two isoforms of COX-1 and COX-2, respectively (Vane *et al*., 1998).

PGs are formed by oxidative cyclization of central five carbons within 20-carbon polyunsaturated fatty acids such as arachidonic acid. The reaction catalyzed by COX includes two key regulatory steps: the enzymatic conversion of the fatty acid to prostaglandin G_2 (PGG_2) and the conversion of PGG_2 to prostaglandin H_2 (PGH_2) by COX (Gupta and DuBois, 2001; Howe *et al*., 2001) (Figure 2.4). First, two oxygen molecules are incorporated into arachidonate to generate PGG_2. In the second step, PGG_2 is reduced to become PGH_2 by peroxidase activity of COX-2. PGH_2 is then converted to one of several PGs, including the PGE_2, PGD_2, PGF_2, PGI_2, and thromboxane A2 (TXA_2), through the function of specific PG synthases.

Two isoforms of COXs have been characterized, COX-1 and COX-2. COX-1 is a housekeeping enzyme which is constitutively expressed in most tissues of body, including epithelial cells of the gastrointestinal tract, while COX-2 is not normally expressed in most tissues and is not detectable in normal epithelial cells of gastrointestinal tract. The constitutive enzyme, COX-1 has noble housekeeping functions such as protecting stomach from ulcers and regulating renal blood flow (Robert, 1983; Whelton, 1999). A wide range of growth factors and pro-inflammatory cytokines in specific pathophysiological conditions induce COX-2 which becomes abundant in activated macrophages and other cells at sites of inflammation (DuBois *et al*., 1994). Increased COX-2 mediated PGE_2 has a strong association with colorectal neoplasia, by promoting cell survival, cell growth, migration, invasion and angiogenesis.

2.6.2 Role of PG in Colon Carcinogensis

Increased concentrations of COX-2 decrease intracellular levels of free arachidonic acid, thus preventing apoptosis (Prescott and Fitzpatrick, 2000). COX-2

enzymatic activity synthesizes PG which alters cell growth, apoptosis, angiogenesis or other mechanisms that lead to cancer. PGE_2 treatment of HCA-7 human colon cancer cell line reduced cells basal apoptotic rate and concomitant increased cellular levels of Bcl-2 (Sheng *et al*., 1998). PGE_2 treatment of another human colorectal carcinoma cell line, LS-174, increased cell motility, cell proliferation, and change in colony morphology (Sheng *et al*., 2001).

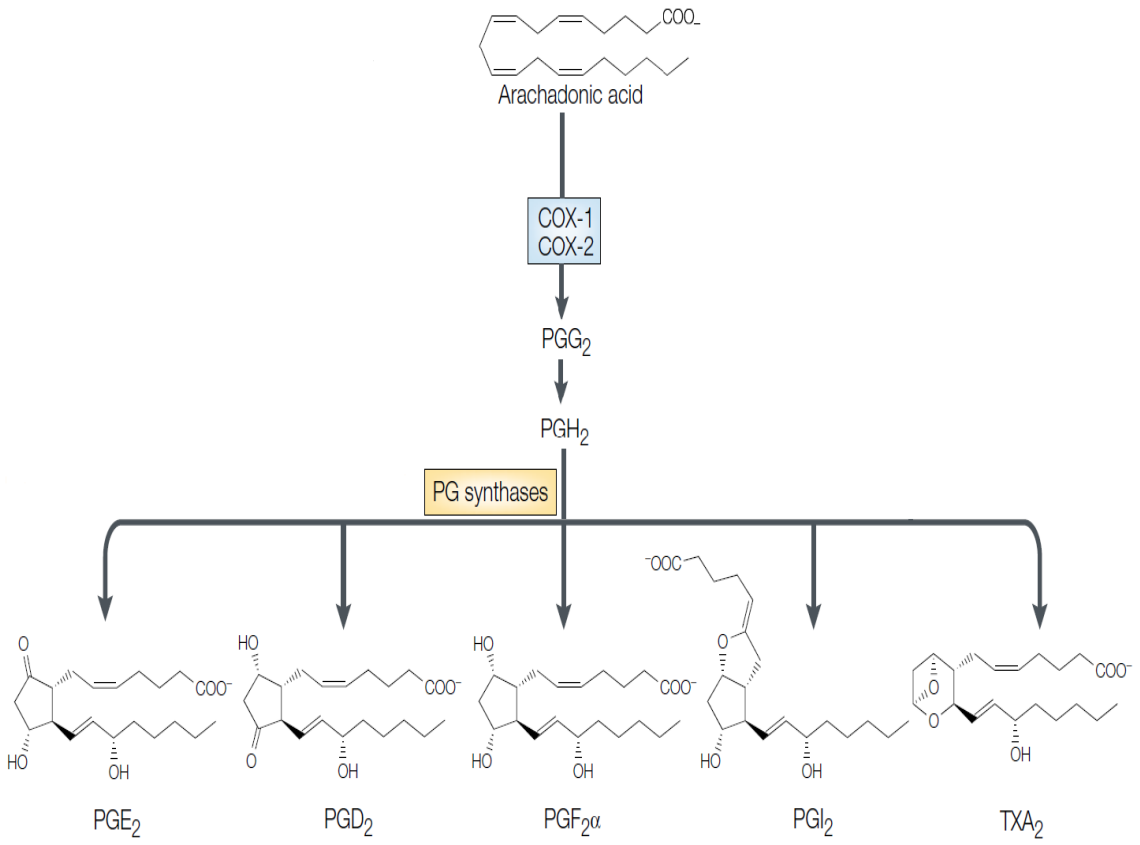


Figure 2.4. Role of COX in prostaglandin synthesis. The COX catalyse biosynthesis of bicylic endoperoxide intermediate PGG2, followed by reduction to PGH2. PGH2 is then converted to one of several PGs, including PGE_2, PGD_2, PGF_2, PGI_2, and TXA_2, through specific PG synthases. (Adapted from Gupta and DuBois, 2001)

This suggested that the PGE_2-induced changes in carcinoma cells are associated with activation of phosphatidylinositol-3′-kinase (PI3K)/AKT pathway caused by activation of PGE_2-receptor subtype EP_4 (Sheng *et al*., 2001). Genetic studies in mice suggest that PGE_2 promotes tumorigenesis at least in part by activating EP_1 receptor subtype. Mice with homozygous deletions in EP_1, but not EP_3, were partially resistant to AOM-mediated induction of aberrant crypt foci (Watanabe *et al*., 1999; Watanabe *et al*, 2000). Moreover, in AOM-treated wild-type mice, an EP_1 receptor antagonist also decreased the incidence of aberrant crypt foci. Finally, Apc^{Min} mice treated with the same EP_1 receptor antagonist had 57% fewer intestinal polyps than untreated mice (Watanabe *et al*., 1999). In contrast, studying the genetic role of all four PGE_2 receptors in development of intestinal polyposis in $Apc^{\Delta 716}$ mice showed that number and size of intestinal polyps was significantly reduced only in mice that harbored a homozygous deletion of the EP_2 receptor (Sonoshita *et al*., 2001). More recently, administration of PGE_2 to $Apc^{Min/+}$ mice has been demonstrated to increase the size and multiplicity of colonic adenomas (Wang *et al*., 2004).

2.6.3 Role of COX-2 in Colon Carcinogensis

Over-expression of COX-2 is a prominent feature in a wide variety of cancers including breast and colon (Eberhart *et al*., 1994; Kargman *et al*., 1995; Sano *et al*., 1995; Liu and Rose, 1996; Kutchera *et al*., 1996, Chan *et al*., 2007). Over-expression of COX-2 in colon cancer cells and during inflammatory processes leads to the over-expression of PGE_2, which can activate E-prostanoid-2 (EP_2) receptors, a G-coupled cytoplasmic PG receptor (Castellone *et al*., 2006) (Figure 2.5). This results in the stabilization and accumulation of β-catenin in cytoplasm and its subsequent translocation to the nucleus,

where it induces the expression of various target genes regulated by the tcf/lef transcription factors. One of the genes up-regulated by this mechanism is Cyclin D1, which is also found to be over-expressed in colon cancer cells, causing cellular proliferation (Tetsu and McCormick, 1999).

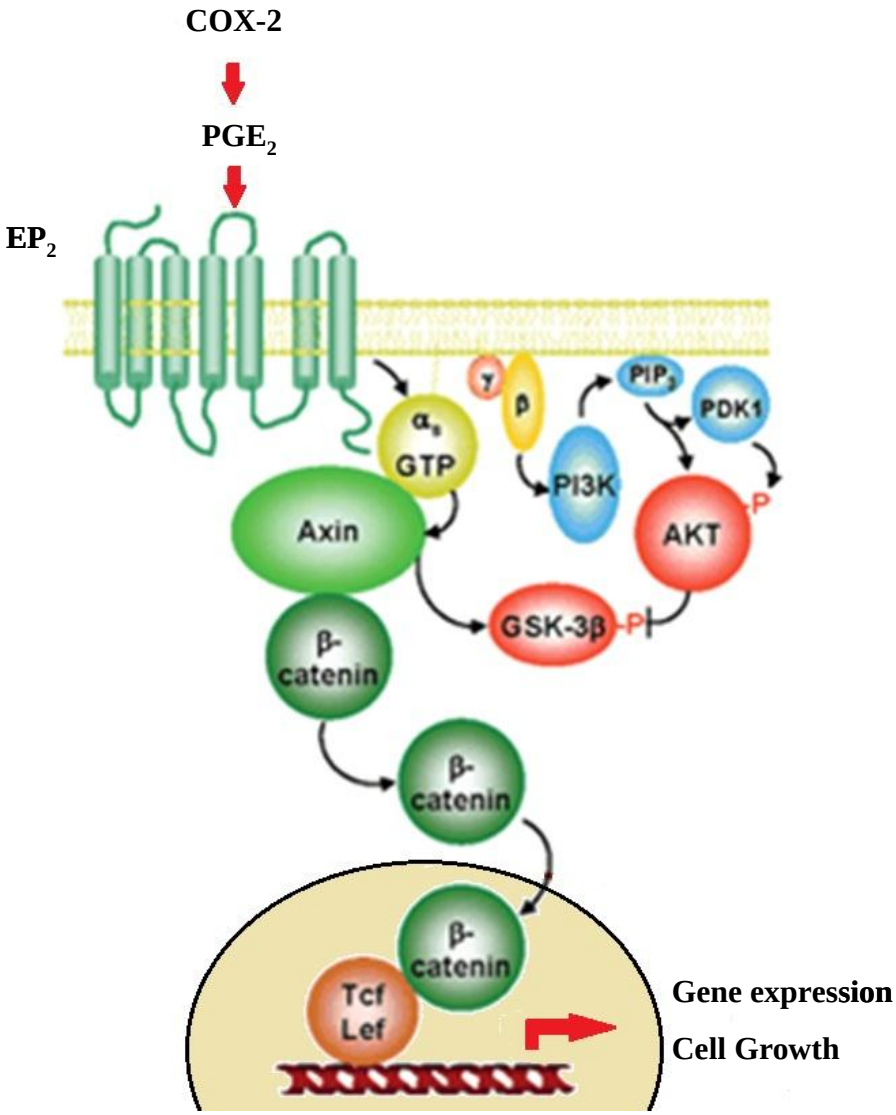


Figure 2.5. The β-catenin pathway provides a link between COX-2 and colorectal cancer. Upon activation of EP_2 receptors by PGE_2, GTP binds to axin, thus promoting the release of GSK-3β from its complex with axin. This causes the accumulation of β-catenin in cytoplasm and its subsequent translocation to nucleus, where it induces the expression of various target genes regulated by the tcf/lef transcription factors. (Adapted from Castellone *et al*., 2006)

Three separate lines of evidences, epidemiological, whole animal pharmacology, and *in vitro* pharmacology, suggest that COX-2 is a rate-limiting step in colon cancer carcinogenesis. Epidemiological evidence shows that individuals who are continuous users of aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) had 40 – 50% decreased mortality from colorectal cancer, suggesting that these drugs may provide a chemoprotective effect against colorectal cancer (Kune *et al*., 1988; Thun *et al*., 1991; Giovannucci *et al*., 1994; Giovannucci *et al*., 1995). In a prospective trial of 635 patients with prior resected early-stage colon cancer, use of chronic aspirin for a mean of 12.8 months showed a 33% reduction of incidence in adenomas compared to control subjects (Sandler *et al*., 2003).

The effect of decreased incidence of colorectal cancer by regular use of various NSAIDs is considered to be by inhibiting COX-2 and, subsequently, prostaglandin synthesis which promotes tissue inflammation, cellular proliferation, and tumor growth (Clevers, 2006). Non-selective NSAIDs inhibit both isoforms of COX-1 and COX-2, but several selective COX-2 inhibitors have been developed to block the development of colonic adenomas and colon cancer (Lichtenstein *et al*., 1995). The epidemiological data derived from separate studies differing in locale, design, and population have consistently shown that regular use of aspirin and other NSAIDs reduce the risk of colon cancer.

However, there is one important qualification to these studies, as aspirin failed to confer any benefit in a randomized trial (Young and Lee, 1999). This observation raises some caution about epidemiological analyses. Celecoxib, a selective COX-2 inhibitor, could both prevent and treat adenomas in Min mouse model of FAP (Jacoby *et al*., 2000). In FAP patients (n = 77) whose disease carried a germline APC mutation, treatment with

Celecoxib (400 mg) twice daily for six months showed a 28% reduction in mean number of rectal polyps (Steinbach *et al*., 2000). Additional studies have shown that another NSAID, Sulindac — a selective COX-2 inhibitor, was also effective in reducing adenoma size and number in patients with FAP (Waddell and Loughry, 1983; Giardiello *et al*., 1993). However, the benefits of Sulindac were not as clear in sporadic colon cancer as in familial colon cancer (Ladenheim *et al*., 1995). These clinical studies support that NSAIDs reduce the risk of developing colon cancer and also explain the mechanism underlying the clinical observations.

COX-2 inhibitors are not free of side effects; therefore, COX-2 inhibition cannot be used as a long-term chemopreventive strategy. COX-2 selective NSAIDs have potential adverse cardiovascular effects, as first discovered by the *Vioxx in Gastrointestinal Outcomes* Research study. This report resulted in the withdrawal of Rofecoxib, a selective COX-2 inhibitor, as it almost doubled the risk of events leading to heart attack or stroke (Malhotra *et al*., 2004; Graham, 2005).

The benefit to risk ratio of a specific NSAID is not conclusive, but there is good indication that in selected doses, a NSAIDs can reduce the effects of certain risk factors predisposing individuals to colon cancer. This is supported by research of NSAIDs in colon cancer animal models. The first direct evidence to establish the role of COX-2 in inactivation and progression of adenomas was provided by testing the role of COX-2 in tumorigenesis in $Apc^{\Delta 716}$ knockout mouse (Oshima *et al*., 1996).

During the first three months after birth, these $Apc^{\Delta 716}$ knockout mice developed hundreds of intestinal polyps. By incorporating the COX-2 null mutation into $Apc^{\Delta 716}$ knockout mice, both number and size of polyps in offspring were decreased up to 86% as

compared to the control animals. Moreover, the reduction was in a gene dosage-dependent manner. This experiment demonstrated that selective genetic elimination of COX-2 protected mice, with a defective APC tumor suppressor, from the development of intestinal tumors. These results were further supported by an observation that a selective inhibitor of COX-2, MF tricyclic also conferred protection against colon cancer (Oshima *et al*., 1996). In conclusion, epidemiological, clinical and animal model studies support that COX-2 isoenzyme contributes to pathogenesis in colon carcinogenesis.

2.7. Programmed Cell Death or Apoptosis

Apoptosis is a complex process characterized by morphological changes such as cell shrinkage, membrane blebbing, chromatin condensation, and nuclear fragmentation (Kerr *et al*., 1972). Cell number regulation between cell proliferation, survival, and death is critical and considered to be one of the important issues for development and maintenance of a multi-cellular organism and in particular for colon (Rupnarain *et al*., 2004). Normally, cells divide and multiply in an orderly manner to produce more cells to adequately meet the body needs by replacing older cells and maintaining body health. Tissue maintenance is a continuous process by which progenitor cells are differentiated into specific cell types to perform specialized functions; however, unwanted cells are shed into intestinal lumen via apoptosis without affecting neighboring cells (Jones and Gores, 1997). This process of cell death (apoptosis) and regeneration is very tightly regulated within intestinal epithelium (Garrison *et al*., 2009). Approximately 15 - 25% of the colonic and rectal epithelium is rejuvenated everyday and the average time for these cells to proliferate is one day. Moreover, the crypt epithelial cells of the entire colon and rectum are replaced every three to four days (Lipkin, 1985). In mice, as the cells reach

villus tip they are shed at a rate of 1,400 cells per villus per day (Potten *et al*., 1997). Therefore, the intestinal epithelium has one of the most rapid turnover rates among mammalian tissues.

In normal colon tissue, stem cells at the bottom of crypts divide continuously in an asymmetric manner and cells are added to the crypt's proliferative zone (Bach *et al*., 2000). These intermediary cells will further differentiate into goblet cells or migrate upward to the lumen (Luebeck and Moolgavkar, 2002). While in malignant tissue, the accumulation of genetic changes disrupts normal division, resulting in symmetric division that both daughter cells may preserve the clonogenic traits of the stem cell. This leads to stem cells alterations and risk of clonal expansion if apoptotic activity is not adequate (Luebeck and Moolgavkar, 2002). As normal colonocytes develop into cancerous cells, there is a change in expression of cell cycle and apoptosis-related proteins (Rupnarain *et al*., 2004).

2.7.1 Apoptosis-Related Proteins: B-Cell Lymphoma 2 (Bcl-2) Families

The mechanism for increased cell population is increased cellular proliferation, and cell survival or decreased apoptosis. The proto-oncogene Bcl-2 inhibits apoptosis and encourages tumor progression (Sinicrope *et al*., 1995), the retinoblastoma protein (RB) regulates cell cycle progression, and the Bax, Bcl-xL, Fas receptor and Fas-ligand, regulate apoptosis and contribute to tumor progression (Backus *et al*., 2002). The induction of apoptosis is a mechanism to protect against colonic DNA damage (Thompson, 1995; Chang *et al*., 1997). There is growing evidence that apoptosis plays an important role in the regulation of cell number and elimination of harmful cells (Hall *et al*., 1994; ; Tomlinson and Bodmer, 1995; Chang *et al*., 1997; Potten *et al*., 1997). The

Bcl-2 family members are intimately involved in apoptosis (Green and Reed, 1998). Therefore, apoptosis related proteins of Bcl-2 family have been of interest to researchers in colon cancer.

Bcl-2 family members are classified into three groups based on BH domains, mitochondrial anchorage (MA), as well as biological action: pro-apoptotic or anti-apoptotic (Budd, 2001). For instance, Bcl-2 and Bcl-xL are anti-apoptotic members which have BH_1, BH_2, BH_3, and BH_4 domains and a carboxy-terminal hydrophobic trans-membrane tail domain to localize the proteins to the outer mitochondrial membrane (Budd, 2001; Mohamad *et al*., 2005). The pro-apoptotic members belong to multi-domain or BH_3-only proteins. Bax and Bak proteins are multi-domain pro-apoptotic members which have BH_1, BH_2, and BH_3 domains and the trans-membrane tail domain (Budd, 2001; Mohamad *et al*., 2005). In addition, Bid, Bad, and Bim are BH_3-only pro-apoptotic proteins which contain only the BH_3 domain and are cytosolic. BH_3-only proteins are required to interact with other Bcl-2 family members such as the multi-domain pro-apoptotic members for oligomerization (Mohamad *et al*., 2005). The molecular mechanisms inducing apoptosis and cell death differ among the Bcl-2 family members due to differences in their structural domains (Reed, 1999). Bcl-2 proteins are present on the outer and inner membrane of the mitochondria where permeability transition pores are located (Tsujimoto, 1989; Reed, 1999). Bax proteins also interact with the same contact sites (Reed, 1999).

The protein members of Bcl-2 family regulate the apoptotic process by promoting or inhibiting permeabilization and disruption of outer mitochondrial membranes (Mohamad *et al*., 2005). Mitochondria release cytochrome c in response to most

apoptosis inducers (such as Bid and Bax) and some anti-cancer drugs by opening channels in the outer membrane (Shanmugathasan and Jothy, 2000; Adrain and Martin, 2001). The change in membrane permeability results in an influx of fluid as well as swelling of organelles and eventual rupture of mitochondria (Green and Reed, 1998). The release of cytochrome c into cytosol results in activation of apoptosis protease-activating factor-1 (Apaf-1), which permits binding of adenosine triphosphate (ATP) and procaspase 9, and triggers its oligomerization to form an apoptosome (Green and Reed, 1998; Adrain and Martin, 2001). The apoptosome cleaves and activates cysteinyl aspartate-specific protease 9 (caspase 9) and initiates the activation of executioner caspases 3, 6, and 7, thus initiating apoptosis. The release of cytochrome c can also be induced by caspase 8 activated Bid and Bax. Bcl-2 or Bcl-xL can suppress the effect of Bax and Bid by preventing the release of cytochrome c or by interfering with the activation of caspase in apoptosome complex.

In crypts of normal epithelium, Bcl-2 is expressed at the highest level at the base and decreases upward along the crypts (McDonnell, 1993), suggesting that the base of crypts has the highest rate of cell division through apoptosis prevention while apoptosis is stimulated as the cells mature along the crypt (Huerta *et al*., 2006). As the colonocytes are genetically altered into carcinomas, apoptosis is gradually decreased (Bedi *et al*., 1995). Similarly, compared with a normal colon, the apoptosis events decreased in patients with FAP, followed by patients with sporadic adenocarcinomas, and carcinomas were found to have the least apoptosis events among the three. The reduction in apoptosis was associated with markedly increased Bcl-2 expression in early carcinomas compared with the normal epithelium. Over-expression of the proto-oncogene Bcl-2

could contribute to tumor progression (Sinicrope *et al*., 1995). Bcl-2 expression increases before the development of morphological dysplasia as Bcl-2 was highly expressed in non-dysplasic epithelium adjacent to dysplasic lesions, suggesting that deregulation of Bcl-2 is an early event in colorectal carcinogenesis (Hague *et al*., 1994; Bronner *et al*., 1995).

Bax is a death promoter and it is inactivated in colon cancer (Rampino *et al*., 1997; Sturm *et al*., 1999). Bax is predominantly localized in cytosol and able to move into the outer mitochondrial membrane in response to an apoptotic stimulus (Mandal *et al*., 1998; Mohamad *et al*., 2005). In the p53 pathway, Bax is a downstream molecule which is mutated in human tumors (Wallace-Brodeur and Lowe, 1999). Bax gene is transcriptionally regulated by p53, and a portion of its tumor-suppressor properties may be mediated by the transcriptional activation of the Bax gene (Sturm *et al*., 1999). In colonic epithelial cells, Bax was found to be highly expressed at the tip of the crypts, where apoptosis is active (Krajewski *et al*., 1994). More than 50% of colorectal cancers with mismatch repair defects have Bax mutations, which abolishes the apoptotic response to chemo-preventive agent such as NSAID (Sturm *et al*., 1999; Zhang *et al*., 2000b). The decreased level of Bax is a negative prognostic factor for patient mortality with liver metastasis and was found to be independent of p53 status (Sturm *et al*., 1999). Best survival rates were seen in patients with wild type p53 and Bax positive-tumors.

2.7.2 Apoptosis Mechanisms in the Unstressed and Stress Induced Intestine

Apoptosis plays an important role in determining the architecture of intestinal epithelia and its stress response to toxic stimuli (Watson and Pritchard, 2000). Apoptosis can be characterized into two broad types: spontaneous apoptosis and stress-induced

apoptosis. Spontaneous apoptosis occurs continuously at low levels in a normal, unstressed intestine and regulates the number of cells entering the crypt/villus axis. Stress-induced apoptosis occurs after genotoxic treatment such as exposure to gamma radiation or DNA-damaging drugs.

Spontaneous apoptosis occurs at the base of a crypt where epithelial stem cells are present and in early transit cells (Watson and Pritchard, 2000). Mice knockout studies have shown that spontaneous apoptosis is independent of p53 and Bax in both small and large intestine epithelium (Clarke *et al*., 1994; Merritt *et al*., 1994). Bax and p53 have little effect on regulation of normal homeostasis in the gastrointestinal tract. On the other hand, in the colon, homozygous Bcl-2 null C57BL/6 mice showed increased spontaneous apoptosis concentrated at the base of a crypt (Merritt *et al*., 1995). In another study, the morphology of colonic epithelium is normal in Bcl-$2^{-/-}$ mice (Nakayama *et al.*, 1993; Nakayama *et al.*, 1994), hence these studies suggest that Bcl-2 plays a role in regulation of spontaneous apoptosis in the normal colonic epithelium.

In contrast, mice knockout (C57BL X DBA/2 F_1) studies have demonstrated that both p53 and Bcl-2 are important regulators of stress-induced apoptosis with significant differences between early and late time points (Clarke *et al*., 1994; Merritt *et al*., 1994). The $p53^{-/-}$ mice did not show intestinal apoptosis normally observed in p53 wild type animals within 3 to 4 hours of gamma radiation damage. Four and half hours after 40 mg/kg of 5-fluorouracil administration, Bcl-$2^{-/-}$ mice showed significantly increased apoptosis predominantly at the base of the colonic crypts (Pritchard *et al*., 1999). In contrast to p53 and Bcl-2, Bax plays only a minor part in the regulation of stress-induced apoptosis. In the same study, $Bax^{-/-}$ mice showed no significant difference in apoptotic

response in small intestine or mid-colon 4.5 hours after gamma radiation or 5-fluorouracil administration as compared with their wild type controls (Pritchard *et al*., 1999). However, a small reduction in apoptotic yield was observed 24 hours after 5-fluorouracil intake. In conclusion, these studies suggest that acute apoptosis is mediated through p53-dependent pathway in early stages after administration of gamma radiation.

2.7.3 Regulation of Apoptosis by APC

Studies of the role of APC in apoptosis for colon cancer have revealed that apoptosis activity may be regulated by the inactivation of APC. In normal human colonocytes, APC expression is frequently found in luminal part of crypt where cells are shedding from the luminal surface after undergoing apoptosis (Midgley *et al*., 1997). The induction of wild type APC in an APC mutant cancer cell line increased cell death via apoptosis, suggesting that APC plays a role in apoptosis regulation (Morin *et al*., 1996), and the loss of function of APC may cause deregulation of apoptosis in colonocytes.

2.8 Mitotic Cell Cycle

Standard eukaryotic cell cycle is divided into four-overlapping phases, with DNA synthesis and mitosis occurring during S and M phase, respectively (Figure 2.6). In G_1 phase and the diploid cell has 2n chromosomes, the cell is growing and it is preparing for DNA synthesis (Figure 2.6). In the subsequent S phase, DNA duplication occurs and at the end of this phase, both DNA content and chromosome number will double (4n) (Figure 2.6). Before cells undergo mitosis, they continue in the G_2 phase with cell growth and are preparing for cell division (Figure 2.6). During mitosis, separation into two daughter cells occurs. Cells which are in G_0 phase (quiescence) are not actively cycling. To avoid inappropriate cell proliferation, control mechanisms are required. The

key regulatory proteins which allow the transition from one cell cycle phase to another are called cyclin-dependent kinases, a family of serine/threonine protein kinases which are activated at specific points during cell cycle. Cyclin D1, one of the cyclins is induced as the G_0 cells are stimulated to enter cell cycle. Defects in cell cycle regulation can result in cancerous growth and developmental abnormalities.

2.8.1 Role of Cyclin D1 in Cell cycle and Colorectal Cancer

Cyclin D1 plays an important role in regulating mitosis, especially progression from the G_1 to S phase of the cell cycle (Arber *et al*., 1997). The cell cycle progresses when an active complex is formed between Cyclin D1 and its binding partners cyclin dependent kinases 4 and 6 (CDK4 and CDK6) through phosphorylation and inactivation of retinoblastoma proteins (RB), which results in activation of transcription factor E2F (Kato *et al*., 1993; Han *et al*., 1999). E2F can then activate expression of genes involved in the S phase progression of cell cycle including cyclin A (Han *et al*., 1999).

In late G_1 phase, there is a defined restriction checkpoint called R-point, after which the cell commits to DNA replication at S phase and finally enters the cell cycle (Pardee, 1989). Before the R-point, cell cycle progression depends on extracellular growth factors; however, after passing the R-point, even in the absence of growth factors the cell cycle continues (Zetterberg *et al*., 1995). Thus, G_1 phase is the decision point in a cell's fate: proliferation, temporary arrest or quiescence, differentiation, apoptosis or senescence. In addition, malfunctioning of a protein involved in G_1/S regulation could be a major cause of malignant transformation. For instance, in human colon cancer malignancies Cyclin D1 transcripts accumulated at the highest level when cells were at mid-G_1 (Arber *et al*., 1997).

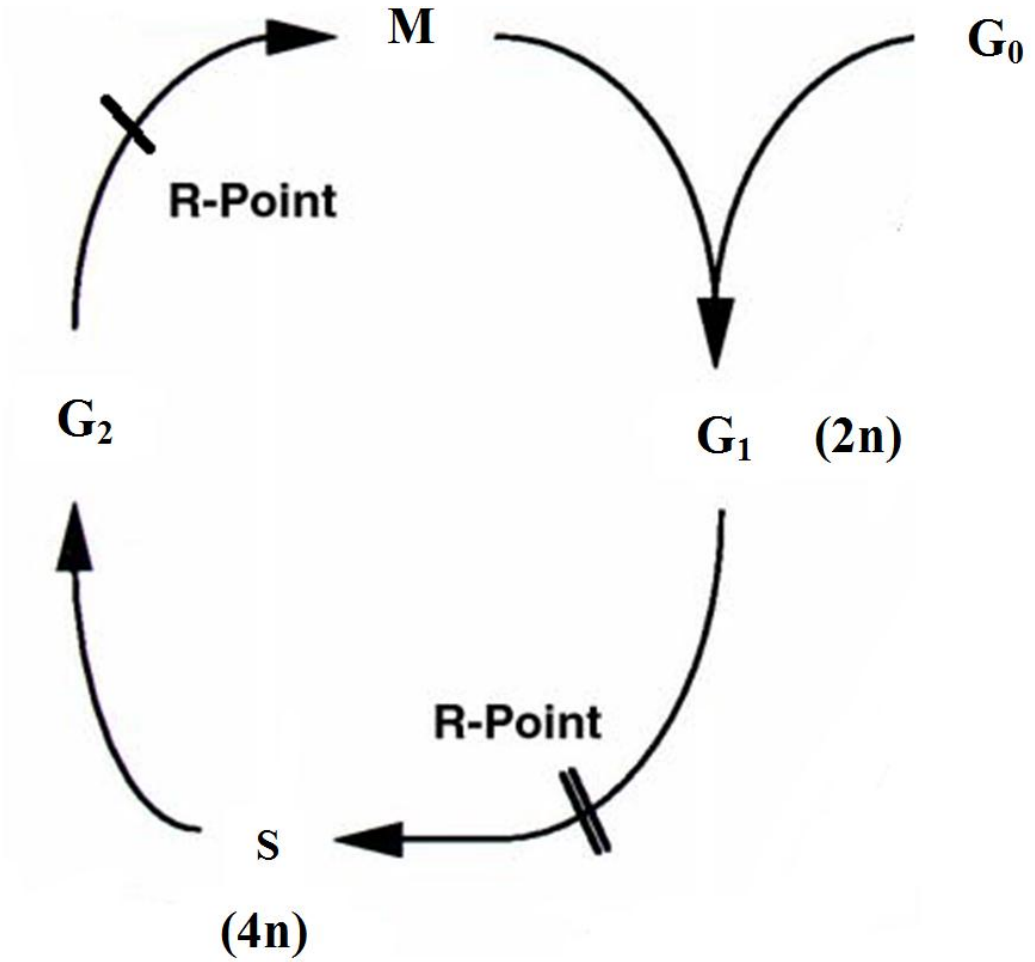


Figure 2.6. The cell cycle clock machinery. G_0, M, G_1, S, and G_2 refer to quiescence, mitosis, first gap, DNA synthesis, and second gap phases of the cell cycle. Cell cycle restriction points (R-points) regulate the complex network of interactions that determine cell growth, cell arrest or apoptosis. (Adapted from Baldassarre *et al*., 2004)

In a normal cell, Cyclin D1 expression can be regulated by APC. Cyclin D1 is one of the downstream targets of the APC/Wnt signalling, in which Cyclin D1 expression is down-regulated by wild type APC and up-regulated by nuclear β-catenin (Saif and Chu, 2010). In addition, Cyclin D1 gene is a direct target for transactivation by β-catenin-lef-1 pathway through a lef-1 binding site in the Cyclin D1 promoter (Shtutman *et al*., 1999). The over-expression of β-catenin caused the induction of Cyclin D1 protein

leads to uncontrollable progression into the cell cycle, thus promoting neoplastic conversion (Shao *et al*., 2005). In contrast, Kazanov *et al*. (2003) did not observe changes in the expression of β-catenin in Cyclin D1 over-expressing cells, indicating that Cyclin D1 is downstream to β-catenin and it does not affect its expression. This could be due to nuclear translocation that may have occurred without affecting overall cellular concentrations.

In several kinds of malignant tumors, such as lung, breast, gastric, esophageal, and colon carcinomas, Cyclin D1 genes are over-expressed and hence called as proto-oncogenes (Arber *et al*., 1996; Arber *et al*., 1997; Dobashi, 2005). A well-characterized human colon cancer cell line SW480E8 with high levels of Cyclin D1 when transfected with anti-sense CyclinD1 cDNA showed an increase in doubling time; a decrease in saturation density, plating efficiency, and anchorage-independent growth; and a loss of tumorigenicity in nude mice. On the other hand, constitutive expression of Cyclin D1 cDNA increased Cyclin D1 causing abnormal growth and tumorigenicity (Arber *et al*., 1997). Moreover, Cyclin D1 protein is found to be over-expressed in nearly 30% of colon adenocarcinomas (Bartkova *et al*., 1994) and 30% of colon adenomatous polyps also show Cyclin D1 over-expression (Arber *et al*., 1996). In another study, a non-tumorigenic intestinal epithelial cell line, IEC-18, when transfected with a vector encoding Cyclin D1, resulted in three clones with Cyclin D1 levels similar to that of colon cancer cell lines proliferated faster than normal cell lines, demonstrated anchorage-independent growth in soft agar, higher saturation density and higher plating efficiency. These Cyclin D over-expressing clones when injected into nude mice, generated tumors within 6-8 weeks (Kazanov *et al*., 2003). This study supports that over-expression of

Cyclin D1 in normal enterocytes resulted in malignant transformation. These observations suggest that Cyclin D1 over-expression is an early event in colon carcinogenesis.

2.9 Role of Inducible Nitric Oxide Synthase (iNOS) in Colorectal Cancer

2.9.1 Nitric Oxide Synthase (NOS)

The NOS isoenzymes family catalyzes the conversion of terminal guanidine nitrogen of amino acid L-arginine to L-citrulline to yield free radical nitric oxide (NO) (Moncada *et al*., 1991). Three distinct isoforms of the NOS enzymes are identified and represent products of three different genes. Two of the NOS enzymes are termed constitutive NOS (cNOS) and are found to be consistently present in the cell while the third enzyme is termed inducible NOS (iNOS), which is not typically expressed in resting cells, but it is expressed in response to cytokines, microbial products, or lipopolysaccharide (Gellar and Billiar, 1998). Thus, iNOS plays an important role in immune response. One of the cNOS was identified in endothelial cells and thus termed as endothelial cNOS (ecNOS) (Garcia-Cardena *et al*., 1996). The second cNOS was found to be localized to neuronal cells (ncNOS) but it is also found to be present in skeletal muscle (Nakane *et al*., 1993). cNOS plays a role in regulation of intracellular signal transduction pathways, hence maintaining cellular physiology (Moncada *et al*., 1991). Although iNOS and cNOS produce NO, iNOS generates micro-molar concentrations of NO which can be sustained over days, compared to the transient, low levels of NO generated by the Ca^{2+}-dependent ecNOS and ncNOS (Gellar and Billiar, 1998).

NO is a short-lived highly-reactive molecule, rapidly oxidized to form stable, inactive end products such as nitrite (NO_2) and nitrate (NO_3) (Moncada *et al*., 1991). The cNOS enzymes are calcium-calmodulin dependent for their activation whereas iNOS is calcium independent (Moncada *et al*., 1991). At high levels of intracellular Ca^{2+}, calcium-calmodulin binding activates cNOS which generates small amounts of NO, which act as messenger molecule and activates soluble guanylate cyclase, thus increasing intracellular cGMP (Moncada *et al*., 1991). The NO generated by NOS enzymes is a potent biological mediator which plays important roles in several physiological and pathopysiological functions such as vasodilation, neurotransmission, and immune response (Moncada *et al*., 1991; Gellar and Billiar, 1998). NO could play a role in carcinogenesis due to several reasons. For example, NO can damage DNA through nitrosation of purines and pyrimidines, which results in hydrolytic deamination of cytosine to uracil and guanine to xanthine (Nguyen, *et al*., 1992); it can interfere with DNA repair (Jaiswal *et al*., 2000), and/or cause post-translational modification such as nitrosylation potentially leading to tumor initiation and promotion/progression (Liu and Hotchkiss, 1995; Wink *et al*., 1998). NO has also been shown to cause DNA strand breaks and mutations in human cells (Geller and Billiar, 1998). Thus, the regulation of iNOS plays a critical role in colon tumorigenesis.

2.9.2 iNOS and Colorectal Cancer

Recent studies have shown increased iNOS expression and enzymatic activity in the breast, head and neck, and colorectal cancers (Thomsen *et al*., 1995; Gallo *et al*., 1998). The iNOS enzymatic activity was found to be markedly higher in about 60% of human colon adenomas and in about 25% of colon carcinomas compared with controls

(Ambs *et al*., 1998). The iNOS enzymatic activity decreased with increasing tumor stages from Dukes A through D and was the lowest in the metastasis to liver and lung. Higher iNOS enzymatic activity was found in cancer specimens than in normal mucosa, and in metastatic tumors than in nonmetastatic tumors (Cianchi *et al*., 2003). These findings suggest that iNOS may contribute to the progression of pathogenesis of colon cancer at the transition of colon adenoma to carcinoma *in situ*.

NO has both anti-tumor and pro-tumor properties. The effect of NO may depend on the timing, concentration, and tissue type (Wink *et al*., 1998). Low concentration of NO stimulates cell growth and protects it from apoptosis, whereas high concentrations of NO inhibits cell growth and induce apoptosis (Kim *et al*., 2001). In contrast, Rao suggested that increased activity of iNOS results in inhibition of apoptosis, which is mediated through inactivation of caspases, nitrosylation of p53 and NFkB and upregulation of COX-2 (Rao, 2004). Several studies have provided evidence for cross talk between COX-2 and iNOS expression and enzyme activities. NO induced COX-2 expression has been observed in conditionally immortalized murine colonic epithelial cells (Mei *et al*., 2000). In animal models of colon cancer, administration of colon-specific carcinogen AOM increases both iNOS and COX-2 enzyme activities in colonic mucosa of F344 rats (Rao *et al*., 1999; Rao *et al*., 2002). Administration of a specific iNOS inhibitor, S, S′-1,4-phenylene-*bis*(1,2-ethanediyl)*bis*-isothiourea (PBIT), in AOM-treated F344 rats resulted in a reduction of AOM-induced aberrant crypt formation by 58% compared with the animals that did not receive the iNOS inhibitor (Rao *et al*., 1999).

In mice with AOM-induced tumors, when treated with iNOS inhibitor L-N(6)-1-iminoethyl)lysine tetrazolamide (SC-51) and AG, the number of tumors was reduced significantly as compared to non-treated controls. An even greater reduction in the number of tumors was observed with the co-administration of SC-51 and the COX-2 inhibitor, celecoxib (Rao *et al*., 2002). Rats fed with 1-N(G)-nitroarginine methyl ester (L-NAME) had a reduction of AOM-induced tumors by 24 to 39% (Kawamori *et al*., 2000). Another iNOS inhibitor 1400W also significant reduced the growth of DLD-2 colon adenocarcinoma cells that were genetically engineered to express iNOS constitutively (Thomsen *et al*., 1997). Similarly, knocking out the iNOS gene in APC^{Min} mice resulted in a marked decreased in the number of intestinal polyps. In the same study, the authors found that administration of the iNOS inhibitor aminoguanidine (AG) in drinking water or an L-arginine-deficient diet to APC^{Min} mice resulted in a significant decrease in adenoma development (Ahn and Oshima, 2001). These observations suggest that iNOS and NO may play an important role in colorectal carcinogenesis. Therefore, developing iNOS and NO inhibitors for the chemoprevention of colon cancer is a mechanistically rational approach.

3.0 HYPOTHESES

1. Phenolic acids present in wheat bran inhibit the growth of colon cancer cells.
2. Colon cancer cell lines can be used to screen the inhibitory effect of phenolic acids.

4.0 THESIS OBJECTIVES

1. To optimize a cell-culture-based screening system to assess the effects of phenolic acids on cell growth.

2. To identify molecular markers for regulation of cell growth, pro-apoptosis, and/or anti-inflammatory activity for individual phenolic acids used in this study.

This study is based on the assumption that diets rich in wheat bran reduce the incidence of colon cancer, thus suggesting that phenolic acids in wheat bran inhibit the growth of colon cancer cells. The present investigation will be limited to study the effect of individual phenolic acids on colon cancer cell lines. Selected diagnostic markers for cell growth, pro-apoptosis and anti-inflammatory process will be analyzed to suggest a possible mode of action of these phenolic acids in colon cancer prevention.

5.0 MATERIALS AND METHODS

5.1 Chemicals and Reagents

All chemicals and reagents used in this study were purchased from Sigma Aldrich (USA) or otherwise noted.

5.2 Phenolic Acids Used in This Study

Eleven phenolic acids previously shown to be present in wheat bran (Verma *et al*., 2009) were used in this study. The diverse phenolic acids are subdivided into two major groups based on the side chain attached to the benzene ring (Kim *et al*., 2006), benzoic acids (Figure 5.1) and cinnamic acids (Figure 5.2). The cinnamic acid derivatives include: caffeic, chlorogenic, ferulic, *p*-coumaric, sinapic, and 3, 4-dimethoxycinnamic acids; and the benzoic acid derivatives include ellagic, gallic, protocatechuiv, salicylic, syringic, vanillic, 2, 5-dihydroxybenzoic, and 4-hydroxybenzoic acid.

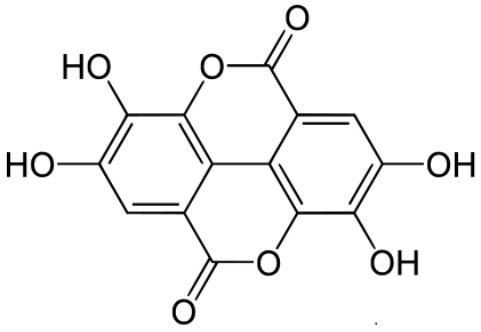
5.3 Human Colon Cancer Cell Culture and Growing Conditions

The human colon adenocarcinoma cell lines HT-29 (ATCC Number: HTB-38) and HCT 116 (ATCC number: CCL-247) used in this study were obtained from the American Type Culture Collection (Rockville, MD, USA). Both cell lines have differences in chromosome numbers and characteristics. HT-29 is a near triploid (modal number of 68 chromosomes) colon cancer cell line with unbalanced translocations and deletions as the most frequent alterations (Camps *et al*., 2004). HT-29 cells display the common chromosomal instability pathway but stable at the microsatellite level (Heinen *et al*., 1995; Camps *et al*., 2004). HCT 116 cells are diploid with modal chromosome number of 46 and show microsatellite instability phenotype (Heinen *et al*., 1995; Ribas *et al*., 2003; Camps *et al*., 2004). HCT 116 cells carry activating mutation in β-catenin gene

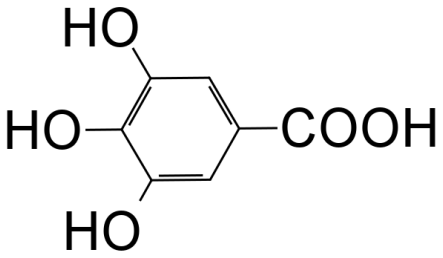
and are wild type for APC (Morin *et al*., 1997), while the undifferentiated HT-29 cells express two C-terminal-truncated APC protein of ~100 and 200 kDa (Hsi *et al*., 1999) and are wild type for β-catenin (Heinen *et al*, 1995; Ilyas *et al*., 1997). In addition, HT-29 cells have normal ras pathway, mutated non-functional p53, low iNOS, and COX-2 positive. HCT 116 cells have oncogene K-ras mutation, high iNOS and COX-2 negative.

HT-29 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma, USA) containing 1mM sodium pyruvate, 1X non-essential amino acids, and 1X vitamins supplemented with 10% (v/v) heat inactivated fetal bovine serum. HCT 116 cells were cultivated in McCoy's 5A medium (Gibco, USA) containing 2 mM glutamine, 10% fetal bovine serum and 1% penicillin and streptomycin. Both cell lines were incubated at 37°C in a 95% humidified atmosphere of 5% CO_2 in the air.

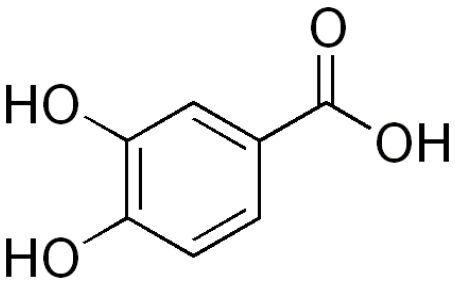
Cells are sub-cultured at pre-confluent densities by using 0.25% trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA) solution. After the old growth medium was aspirated, cells were washed with phosphate-buffered saline (PBS, pH 7.4) solution. Eight millilitres of 0.25% trypsin-EDTA solution was added into each flask and swirled liquid around for 15 seconds. After the trypsin-EDTA solution was aspirated, flasks were transferred to 37°C incubator for about 5 minutes. Cells were then observed under a reverse-light microscope at the end of the incubation period. Ten millilitres of complete medium with serum was added after gently shaking or tapping the edge of the flask to loosen the cells attached at the bottom of the flask. Cells were then re-suspended by taking up the medium into a 10 mL sterile pipet several times in order to dislodge the cells from the flask. One milliliter of re-suspended cells was transferred to



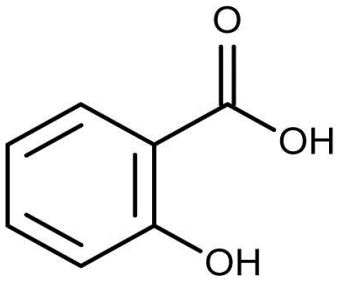
(a) Ellagic acid
(4,4',5,5',6,6'-Hexahydroxydipheric acid)



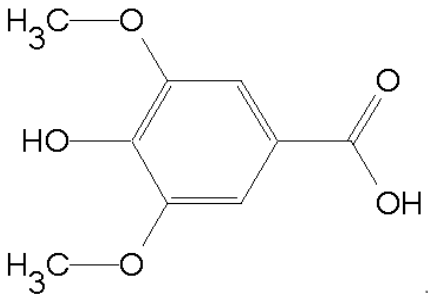
(b) Gallic acid
(3, 4, 5-Trihydroxybenzoic acid)



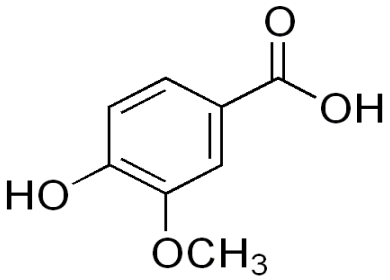
(c) Protocatechuiv acid
(3, 4-Dihydroxybenzoic acid)



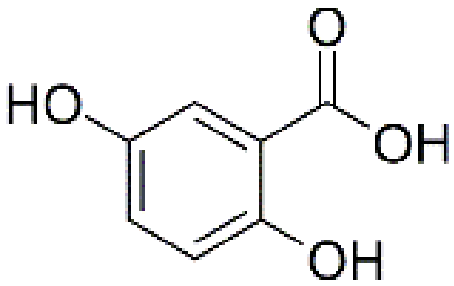
(d) Salicylic acid
(2-Hydroxybenzoic acid)



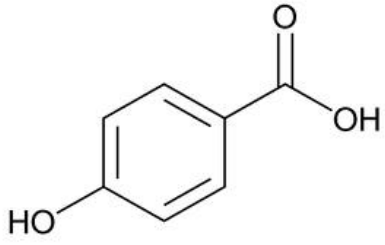
(e) Syringic acid
(3,5-Dimethoxy-4-hydroxybenzoic acid)



(f) Vanillic acid
(3-Methoxy-4-hydroxybenzoic acid)

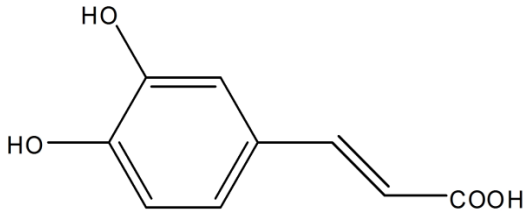


(g) 2, 5-Dihydroxybenzoic acid
(Gentisic acid)

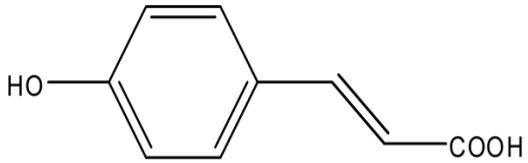


(h) 4-Hydroxybenzoic acid
(*p*-Hydroxybenzoic acid)

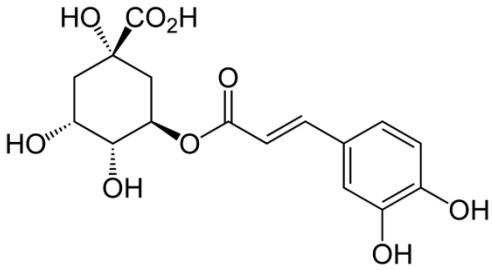
Figure 5.1. Chemical structures of benzoic acid derivative phenolic acids used in this study.



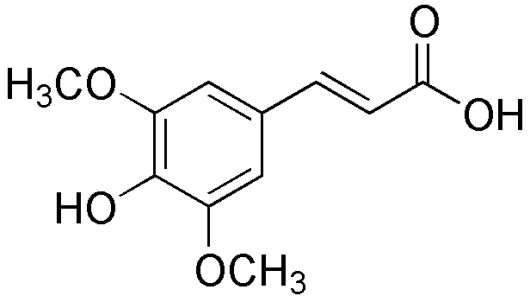
(a) Caffeic acid
(3, 4-Dihydroxycinnamic acid)



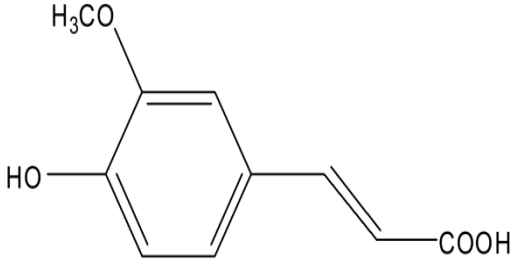
(d) *p*-Coumaric acid
(Trans-4-hydroxycinnamic acid)



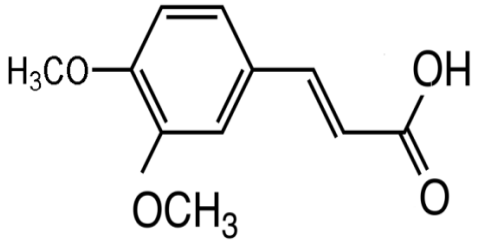
(b) Chlorogenic acid
(1, 3, 4, 5-
Tetrahydroxyclohexanecarboxylic acid)



(e) Sinapic acid
(3, 5-Dimethoxy-4-hydroxycinnamic
acid)



(c) Ferulic acid
(Trans-4-hydroxy-3-methoxycinnamic
acid)



(f) 3, 4-Dimethoxycinnamic acid
(Caffeic acid dimethyl ether)

Figure 5.2. Chemical structures of cinnamic acid derivative phenolic acids used in this study.

a new flask followed by adding 5 mL fresh complete medium with serum.

5.4 Cell Culture Based Screening Assay

5.4.1 Cell Treatment with Phenolic Acid Compounds

All phenolic acid compounds were dissolved in dimethyl sulfoxide (DMSO) solution, except ferulic and vanillic acids, which were dissolved in double distilled water. For testing, HT-29 and HCT 116 cells were seeded at a density of 6×10^5 cells/flask in T-25 cell culture flasks with DMEM or McCoy's 5A medium, respectively. After 24 hours, growth medium of each flask was aspirated and replaced with freshly-prepared complete medium containing phenolic acid compound of 0, 10, 100, 200, 400 and 800 μg/mL concentrations. Three flasks of cells were grown for each set of phenolic acid concentration treatment. In total, there were six groups of concentration treatments. After labeling each flask, flasks were transferred to 37°C incubator for 48 hours.

5.4.2 Cell Harvest and Agar-Cell-Pellet Blocks

After 48 hours of incubation with phenolic acids, cells' morphology and percentage of confluence were examined under a reverse phase-light microscope. After discarding the culture medium, cells at the bottom of each flask were loosened by using Dulbecco's phosphate-buffered saline (D-PBS, pH 7.4) containing 2 mM EDTA but without Ca^{2+} and Mg^{2+} as the regular 0.25% trypsin-EDTA solution would alter the cell morphology or damage the cells prior to immunohistochemistry assay. After 5 minutes incubation in 37°C incubator, 1.5 mL of fresh medium were added to each flask and cells were re-suspended by pipetting up and down for several times. The 1.5 mL medium from the first flask were then transferred into the second flask of that particular set of concentration treatment and cells were re-suspended again by pipetting up and down for several times.

The 1.5 mL medium from the second flask was then transferred into the third flask of the particular set of concentration treatment and cells were re-suspended by pipetting up and down several times. Cells were collected as much as possible from the third flask and were transferred to a new 1.5 mL microcentrifuge tube. Cells were gently centrifuged down at 450 x g for 3 minutes and the supernatant was discarded. In order to mix the cells with 10% melted bactoagar better, the microcentrifuge tube was gently flicked with an index finger tip to loosen the cells at the bottom. A sterile transfer pipette was then used to add about 0.3 ml of 10% melted bactoagar into each microcentrifuge tube. Cells and bactoagar were quickly mixed together using a pipette tip and were centrifuged for about 10 seconds. Microcentrifuge tubes were filled with 10% buffered formalin solution on top of it and stored overnight at 4°C to fix the cells in the bactoagar. After 24 hours, buffered formalin solution was aspirated and the agar-cell-pellet was scooped out from the microcentrifuge tube using a small spatula and placed them on a histology cassette lined with tissue paper cut to fit in both sides of a grid of the cassette. The agar-cell-pellets were divided with several pieces of normal liver tissues to keep the agar separately and aligned in the cassette. Different tissue color dyes were used to add on the agar-cell-pellets to help to maintain the orientation of the agar-cell-pellets. A cassette was placed in a container filled with 10% buffered formalin solution and then embedded with paraffin using standard procedures. Serial 4-μm-thick sections were examined after staining with hematoxylin and eosin.

5.5 Cell Viability

5.5.1 Trypan Blue Exclusion Method

The cell viability counting was performed using the Trypan Blue Exclusion method (Strober, 1997) and hemocytometer under a reverse light microscope at a magnification of 20X.

5.6 Immunohistochemistry Analysis

5.6.1 Immunohistochemical Staining

Immunohistochemical staining was performed using Dako autostainer. Serial 4-μm-thick sections cut onto positively-charged slides were examined from the formalin-fixed and paraffin-embedded agar-cells-pellets, dried, and baked overnight at 60°C. Slides were deparaffinized with xylene and dehydrated by ethanol. After washing with double-distilled water, sections were immersed in freshly-prepared 3% hydrogen peroxide (H_2O_2) solution dissolved in absolute methanol at room temperature for 10 minutes to block endogenous peroxidase activity which will cause false positives in staining methods. The deparaffinized sections were washed three times with double distilled water for 5 minutes, and then stored in fresh double-distilled water until next step.

The microwave method was used in the heat-induced epitope retrieval step to ensure the removal of cross bridges formed in formalin fixation. Slides were microwaved in 1mM EDTA buffer (pH 9.0) for 13 minutes at power of level 6 (1200W) and cooled in the buffer tray for 20 minutes. Slides were placed and cooled under running distilled water to rinse off remaining EDTA. After cooling for another three minutes, slides were immediately used for staining. Immunohistochemical staining was performed using Dako autostainer and the Envision plus kits and reagents (Dako Canada Inc. ON, Canada). The sections were incubated with mouse anti-human Bax monoclonal antibody (Clone 2D2, Thermo Fisher Scientific, Freemont, CA, USA) at 1:150 dilution; mouse anti-human monoclonal Bcl-2 (Clone 124, Dako Cytomation Inc., Carpinteria, CA, USA) at 1:100 dilution; mouse anti-human β-catenin monoclonal antibody (clone beta catenin-1; Dako Cytomation Inc., Carpinteria, CA, USA) at 1:200 dilution; mouse anti-human COX-2 monoclonal antibody (Clone CX-294; Dako Cytomation Inc., Carpinteria,

CA, USA) at dilution 1:50; rabbit anti-human Cyclin D1 monoclonal antibody (Clone SP4, Neomarkers Lab Vision, Freemont, CA, USA) at 1:100 dilution; purified mouse anti-human iNOS (Clone6/iNOS/NOS type II, BD Transduction Laboratories, Lexington, KY, USA) at 1:40 dilution or mouse anti-human monoclonal Ki67 (Clone MIB-1, Dako Cytomation Inc., Carpinteria, CA, USA) at 1:50 dilution. Once the staining was completed, slides were immediately placed in distilled water and were toned by incubating the slides in 2% copper sulphate solution for 5 minutes. After toning, slides were rinsed quickly with distilled water, washed for 5 minutes, and then counter stained with light haematoxylin 1 (10 to 15 dips maximum) followed by rinsing with distilled water until slides were completely clear. Slides were then dehydrated by ethanol and stored in final xylene wash until a coverslip was mounted onto the sections with a few drops of xylene compatible mounting solution.

5.6.2 Evaluation of Immunostaining

After immunostaining, Aperio ScanScope CS Slide Scanner (Aperio Technologies Vista, VA, USA) system was used to capture complete slide digital images with a 20X objective. Three hundred cells were randomly selected from the scanned slide for immunohistochemical analysis. Staining intensities in each group of cell treatment were evaluated by using the DMSO control as internal built-in controls. The staining intensity and the percentage of tumor cells stained were analyzed. Based on staining intensity, scores were assigned as 0 (negative), +1 (weak staining), +2 (medium staining), and +3 (strong staining). Results are scored by multiplying the percentage of positive cells by the intensity. A combined final score was obtained by multiplying cell numbers with staining intensity.

5.7 Western Blot Analysis

5.7.1 Sample Preparation and Protein Extraction

HT-29 and HCT 116 cells were seeded at a density of 6×10^5 cells/flask in six T-25 cell culture flasks with DMEM or McCoy's 5A culture medium, respectively. After 48 hours incubation with test phenolic acid compounds at 0, 10, 100, 200, 400 and 800 μg/ml concentrations, the supernatant was removed from each flask. The attached cells were washed twice with 5 ml ice-cold D-PBS solution before protein extraction. Cells were removed from the cell culture flask by gently scraping with a cell-scraper followed by centrifugation of the cell suspension for 5 minutes at 450g in a pre-cooled (4°C) centrifuge. Cellular proteins were extracted with the Mammalian Cell Lysis Buffer containing 1 U Benzonase nuclease and 1X protease inhibitor (Qiagen, Canada). Lysates were clarified by centrifugation at 14 000g for 10 min at 4°C and the supernatant containing cell lysate was transferred into a fresh, pre-cooled 1.5 ml microcentrifuge tube to be stored at -20°C.

5.7.2 BCA Protein Quantitation

Total protein concentration in the samples was determined using a BCA assay kit (Smith *et al*., 1985; Pierce Biotechnology, Rockford, IL, USA).

5.8 SDS-PAGE and Western Blotting

Samples containing 10 to 30 μg of total protein were mixed with 10X of sample buffer (Laemmli, 1970) containing 2.3% (w/v) SDS, 10% (w/v) glycerol, 5% (w/v) 2-mercaptoethanol, 0.005% (w/v) bromphenol blue and 62.5 mM tris HCl, pH to 6.8 with HCL (Sigma, UK). The samples were then denatured by heating at 95°C for 4 minutes. Twelve percent of sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate the polypeptides, which were subsequently electro-blotted onto polyvinylidene difluoride (PVDF)

membrane (Bio-Rad, Mississauga, ON, Canada) in a transblot electrophoretic transfer cell (Bio-Rad) in transfer buffer.

5.9 Immunodetection

After transblotting, the membranes were blocked with 3% (w/v) non-fat dry milk (Safeway skimmed milk powder) dissolved in 1X TBST (100 mM Tris pH 8, 150 mM NaCL, 0.05% Tween 20) for 2 hours at room temperature. Membranes were incubated with mouse anti-human Bax monoclonal antibody (Thermo Fisher Scientific, Freemont, CA, USA) at 1:1000 dilution, rabbit anti-human Cyclin D1 monoclonal (Neomarkers, Lab Vision, Freemont, CA, USA) at 1:500 or mouse anti-human β-actin monoclonal antibody (Sigma, USA) at 1:5000 dilution for overnight at 4°C in a cold room. After incubation, membranes were washed four times for 10 minutes each in Tris-buffered saline, containing 0.05% Tween 20. The washed membranes were incubated with either goat anti-mouse IgG or goat anti-rabbit IgG antibodies conjugated with horse radish peroxidase (Santa-Cruz, USA) at 1:1000 dilution for 1.5 hours at room temperature. After incubation, the membranes were washed four times for 10 minutes each, and subsequently incubated with luminous ECL Plus reagent (Pierce Chemical, Rockford, IL, USA) for 20s and exposed to Kodak X-ray film (Fuji, Japan) for 1s to 10 minutes. The X-ray films were scanned and the densities of the protein bands were analyzed with Adobe Photoshop software (version 7.0).

6.0 RESULTS

6.1 Sensitivity of Individual Phenolic Acids on Cell Proliferation

To develop a cell culture based screening assay for wheat bran constituents, two colon cancer cell lines were exposed for 48 hours to individual phenolic acids in concentrations ranging from 0 (untreated control) to 800 μg/mL. Cell viability assessment using Trypan Blue Exclusion method showed that in both the cell lines HT-29 and HCT 116 almost all phenolic acids reduced cell proliferation in a concentration dependent manner; however, the degrees of inhibition were different for each phenolic acid.

6.2 HT-29 Cell Viability

Both cinnamic acid and benzoic acid derivatives reduced cell viability at higher concentrations; however, cell survival was different for each phenolic acid. Among the cinnamic acid derivatives, caffeic acid at 10 μg/mL reduced cell viability to 38% of untreated cells (Figure 6.1). However, increasing concentrations of caffeic acid caused only minor reductions in cell viability. Chlorogenic acid showed a gradual concentration dependent reduction in cell viability with only 10% cells were viable at highest concentration (800 μg/mL). The other four phenolics in the cinnamic acid group did not have a significant effect on cell viability at lower concentrations; higher concentrations (400 and 800 μg/mL) reduced cell viability to 25 to 40% of untreated controls (Figure 6.1).

In the benzoic acid-derived phenolic acid group, gallic acid reduced cell viability in a concentration-dependent manner with only 14% cells being viable at the highest concentration (800 μg/mL). All other phenolic acids only marginally reduced cell growth at lower concentrations (10 and 100 μg/mL), except vanillic acid, which at higher concentrations reduced cell viability to around 50% of the untreated control cells (Figure 6.2).

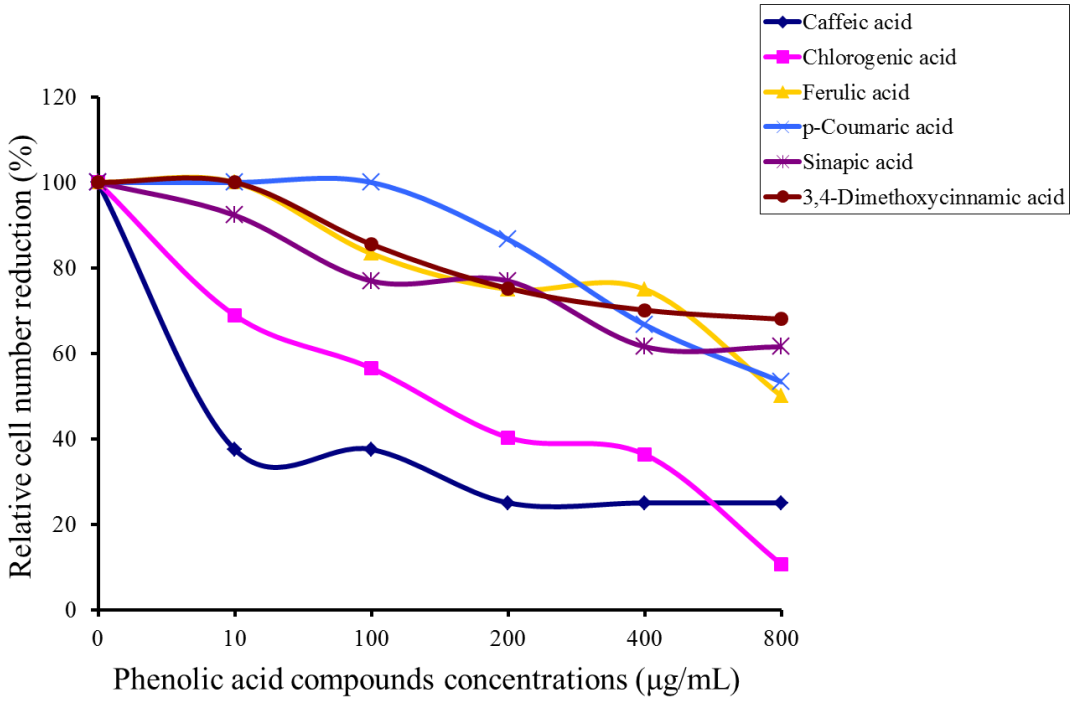


Figure 6.1. Effect of cinnamic acid derivative phenolic acids on HT-29 colon cancer cell growth. The cell number reduction was expressed as relative percentage and the phenolic acid concentrations used were range between 0 and 800 µg/mL.

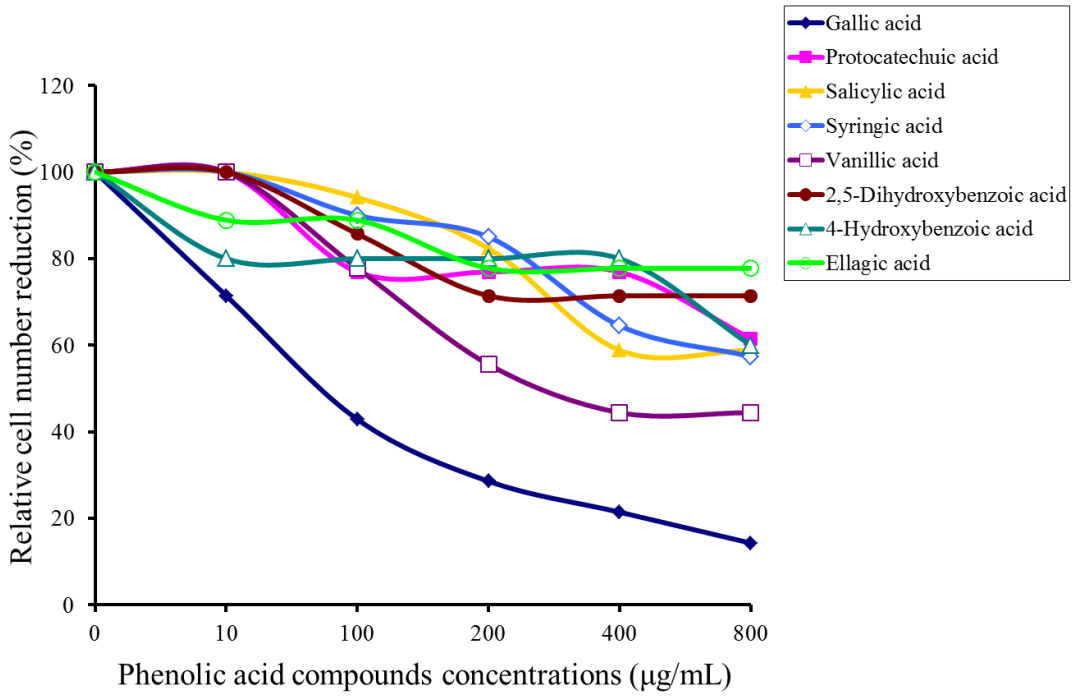


Figure 6.2. Effect of benzoic acid derivative phenolic acids on HT-29 colon cancer cell growth. The cell number reduction was expressed as relative percentage and the phenolic acid concentrations used were range between 0 and 800 µg/mL.

6.3 HCT 116 Cell Viability

Most of the phenolic acids at higher concentrations decreased the growth of HCT 116 cell line. However, compared to HT-29, HT 116 behaved in a different manner, as at lower concentrations some of the phenolics stimulated cell survival compared to untreated control cells. In the cinnamic acid-derived group, caffeic acid at 10 μg/mL reduced cell numbers to 82%; but at 100 μg/mL, only 17% cells survived and at 400 and 800 μg/mL, no viable cells were detected (Figure 6.3). The other phenolic acids in this group at lower concentrations (10 and 100 μg/mL) did not have inhibitory effect on cell survival or proliferation, but at higher concentrations decreased cell number, with the most effect observed at 800 μg/mL. Sinapic acid was the only exception, which at higher concentrations had very little inhibitory effect on cell survival or proliferation (Figure 6.3).

In the benzoic acid derivatives, gallic acid, protocathechuic acid and 2, 5-dihydroxybenzoic acid significantly increased cell numbers at the lowest concentration (10 μg/mL) (Figure 6.4). In gallic acid a sharp decline in cell survival was observed at 100 μg/mL, no cells survived and it stayed the same at higher concentrations. Protocatechuic acid showed slight increase at 100 μg/mL, but at 200, 400 and 800 μg/mL the cell numbers gradually reduced in a concentration-dependent manner reaching almost zero cell survival at the highest concentration. Ellagic acid showed a gradual concentration-dependent reduction in cell survival and at 800 μg/mL no viable cells were detected (Figure 6.4). All other phenolic acids in this group had very little or no effect on cell survival or proliferation. 2, 5-Dihydroxybenzoic acid was unique as it showed a sigmoid curve in cell survival and proliferation, at 10 μg/mL a significant increase - at 100 μg/mL, a decrease, but again with increasing concentrations gradual increase in cell survival and proliferation as compared to untreated control cells (Figure 6.4).

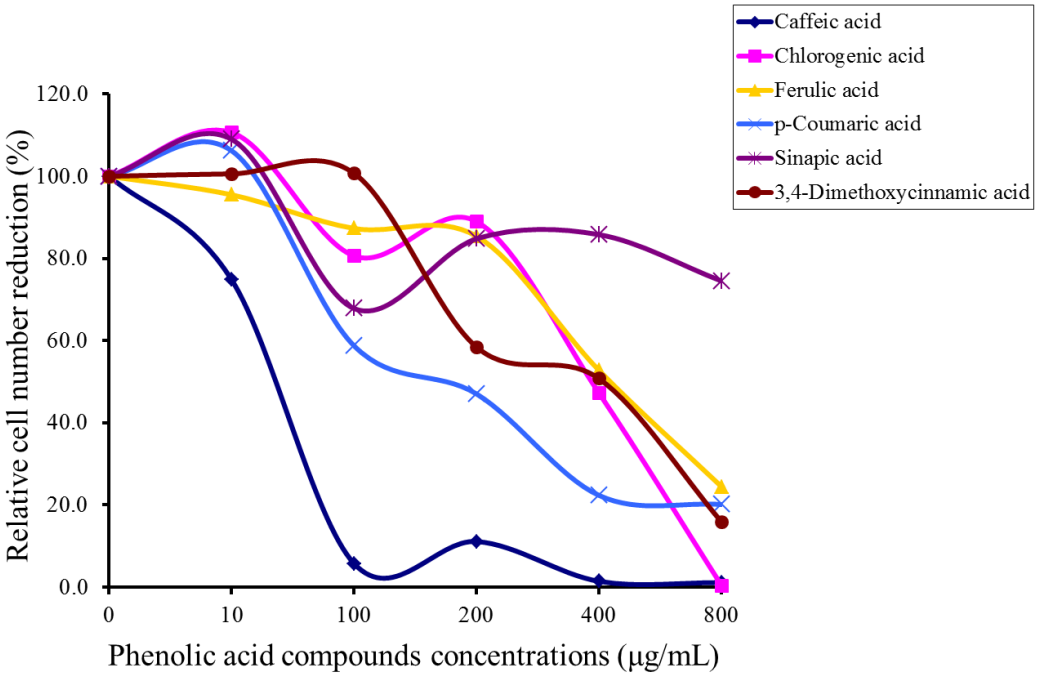


Figure 6.3. Effect of cinnamic acid derivative phenolic acids on HCT 116 colon cancer cell growth. The cell number reduction was expressed as relative percentage and the phenolic acid concentrations used were range between 0 and 800 µg/mL.

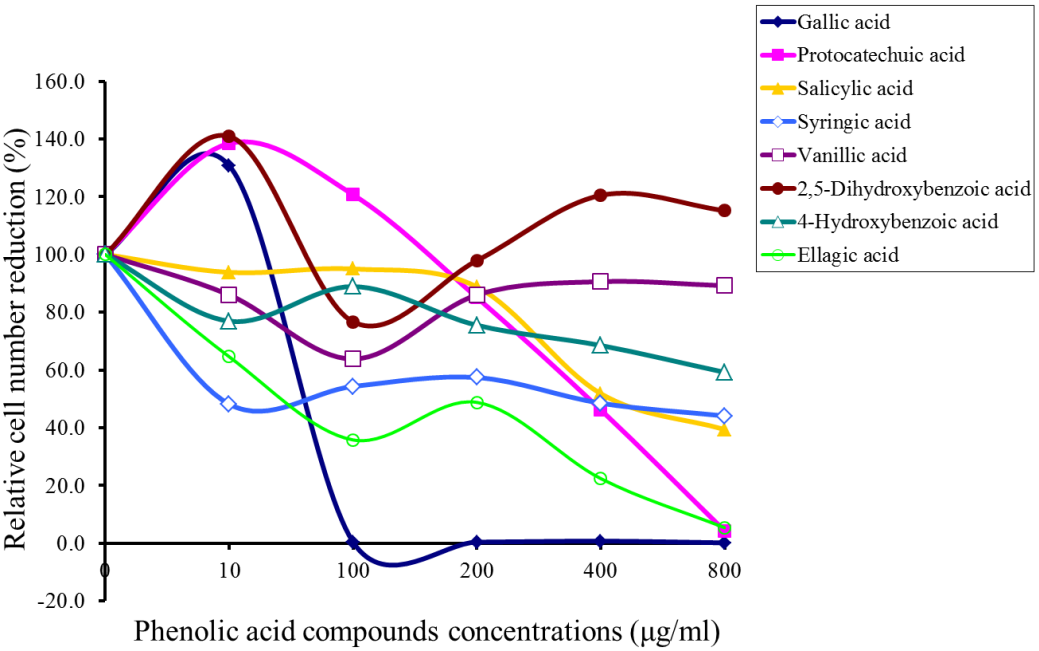


Figure 6.4. Effect of benzoic acid derivative phenolic acids on HCT 116 colon cancer cell growth. The cell number reduction was expressed as relative percentage and the phenolic acid concentrations used were range between 0 and 800 µg/mL.

After screening the efficacy of all fourteen phenolic acids on cell survival, proliferation, and their effect on expression of protein markers related to cell proliferation, apoptosis, and inflammatory pathway, we determined half maximal inhibitory concentration (IC_{50}) for selected phenolic acids. Caffeic and Gallic acids were tested on both the cell lines HT-29 and HT116, while ellagic acid with only HCT 116 cells and chlorogenic acid with only HT-29 cells.

The two phenolic acids, caffeic and gallic acids showed differential effects on both the cell lines. In HT-29 cells, caffeic acid at 10 μg/mL decreased cell viability to 40%, but with increasing concentrations did not show any further reductions in cell viability compared to non-treated controls. In HCT 116 cells, caffeic acid showed a gradual concentration-dependent decrease in cell viability, and at 400 and 800 μg/mL, no viable cells were detected (Figure 6.5). On the other hand, with HCT 116 cells, gallic acid (10 μg/mL) showed 20% increase in cell viability compared to non-treated control cells, but at 100 μg/mL or higher concentrations, no viable cells were detected (Figure 6.5). HT-29 cells treated with gallic acid showed a concentration dependent gradual decrease in cell viability, which at 800 μg/mL showed only 20% viable cells compared to non-treated control cells (Figure 6.5). HCT 116 cells were treated with ellagic acid and it showed in a concentration-dependent gradual reduction in cell survival. Similarly in HT-29 cells, chlorogenic acid showed a gradual concentration-dependent gradual decrease in cell survival, and at 800 μg/mL only 40% of cells were viable compared to untreated control cells (Figure 6.5).

6.4 Immunohistochemical Analysis

To characterize the possible mode of action of phenolic acids in colon cancer prevention, phenolic acid-treated cells for both cell lines (HT-29 and HCT 116) were immunohistochemically analyzed for seven protein markers: Ki67 and Cyclin D1 for cell

proliferation, Bax (pro-apoptotic) and Bcl-2 (cell survival) for apoptosis and COX-2, iNOS, and β-catenin for inflammatory pathway. All the antibodies showed positive reaction with the respective proteins, however Bcl2 showed a very weak reaction as compared to the other six marker proteins in this study. Therefore, Bcl-2 was not included for quantitative analysis.

Representative photographs of histological sections of human colon carcinoma cell line HT-29 and HCT 116 treated with chlorogenic acid and ellagic acid respectively were shown in Appendix 1 – 12.

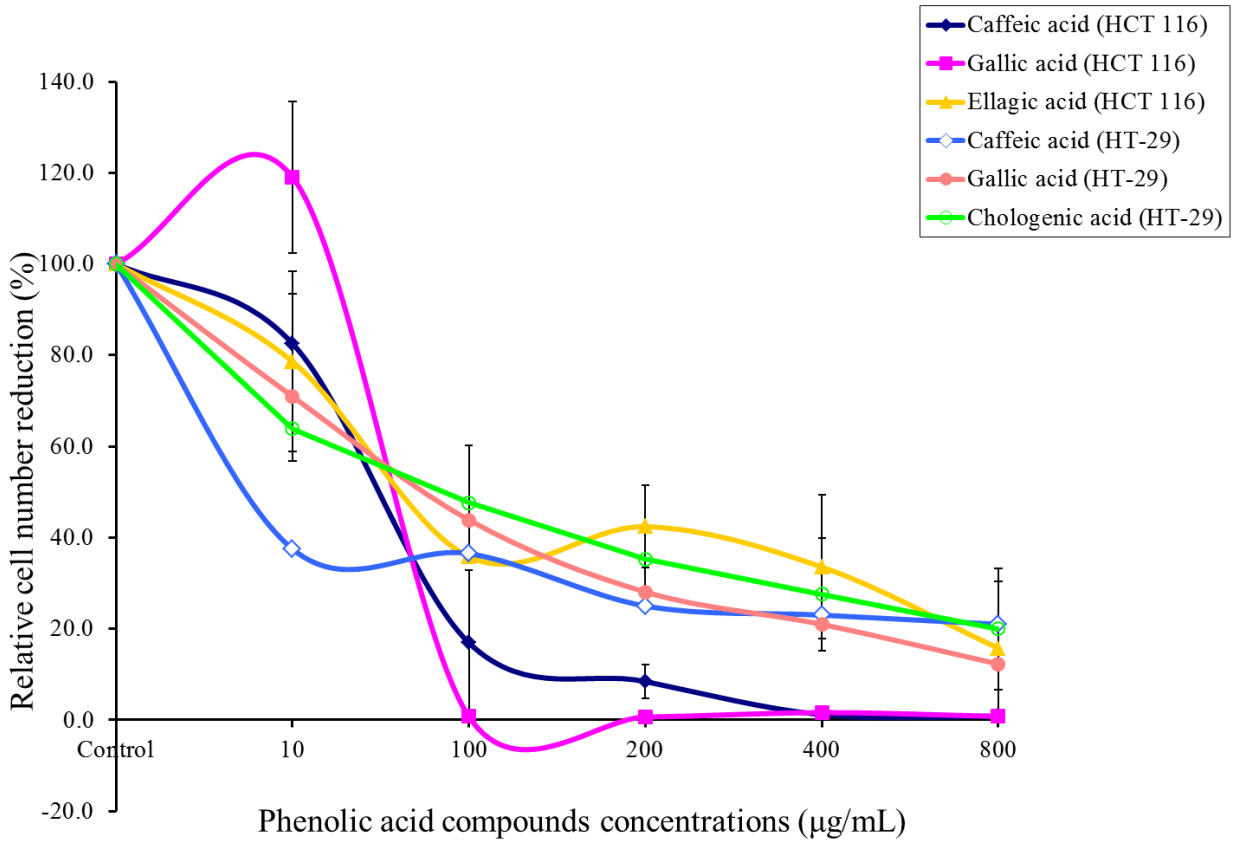


Figure 6.5. The growth inhibitory effect of phenolic acids on HCT 116 and HT-29 cells.

6.5 Modulation of Marker Proteins by Cinnamic Acid Derived Phenolic Acids on HT-29 Cell Line

6.5.1 Caffeic Acid

The two cell proliferation markers, Cyclin D1 and Ki67, both showed slight reductions in response to increasing phenolic acid concentration (Figure 6.6). The reduction was more in Cyclin D1 and less in Ki67; however, neither marker showed any significant decrease in staining intensity. The apoptosis marker, Bax showed a gradual concentration-dependent increase in expression, increasing to 1.20-fold, 1.55-fold, and 1.82-fold at 10 μg/mL, 100 μg/mL, and 200 μg/mL of caffeic acid respectively. Among the three inflammatory markers studied, β-catenin and iNOS showed a gradual decrease, with β-catenin levels showing higher reduction than iNOS. The COX-2 marker was slightly increased in caffeic acid treated cells, with 100 μg/mL showing the most increase.

6.5.2 Chlorogenic Acid

The cell proliferation marker Cyclin D1 showed a gradual decrease with increasing chlorogenic acid concentration, decreasing to 0.81-fold, 0.75-fold, 0.59-fold, and 0.63-fold at 10 μg/mL, 100 μg/mL, 200 μg/mL, and 400 μg/mL, respectively (Figure 6.7). The Ki67 did not show a consistent reduction in expression with increasing chlorogenic acid concentrations. The apoptosis marker Bax increased 1.11-fold, 1.16-fold, 1.44-fold, and 1.47-fold at 10 μg/mL, 100 μg/mL, 200 μg/mL, and 400 μg/mL of chlorogenic acid. Among the inflammatory markers, no consistent trend was observed in HT-29 cells in response to chlorogenic acid treatment. The very high level of iNOS in response to 200 μg/mL is considered to be an aberration.

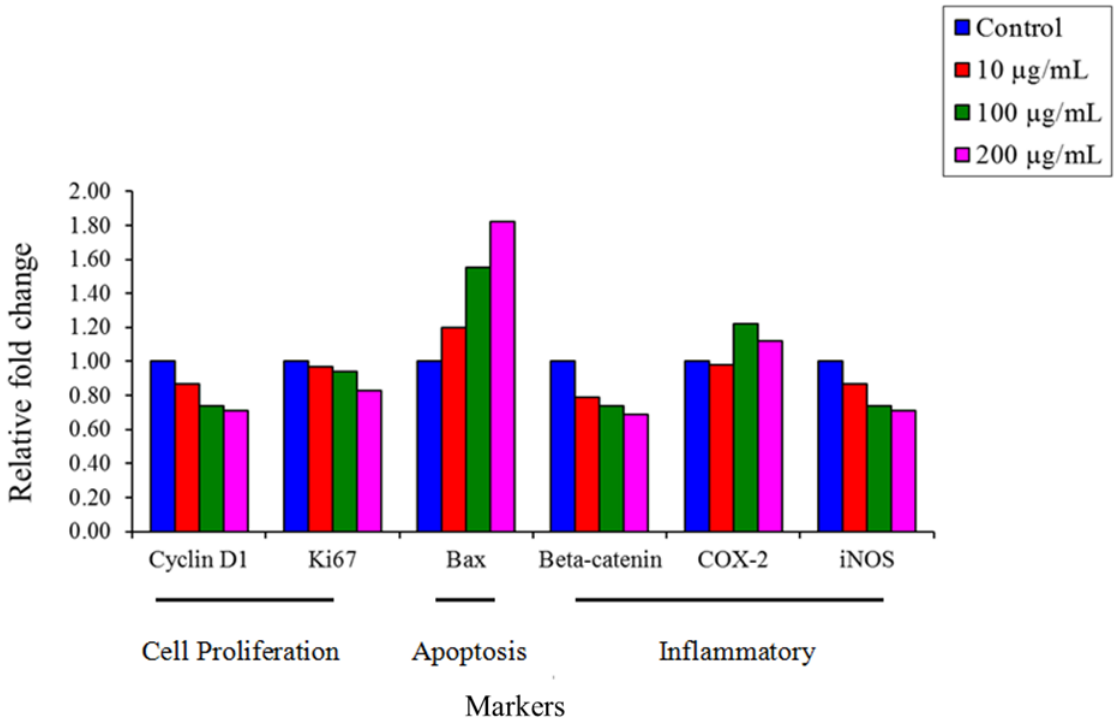


Figure 6.6. Effect of caffeic acid on HT-29 cell line.

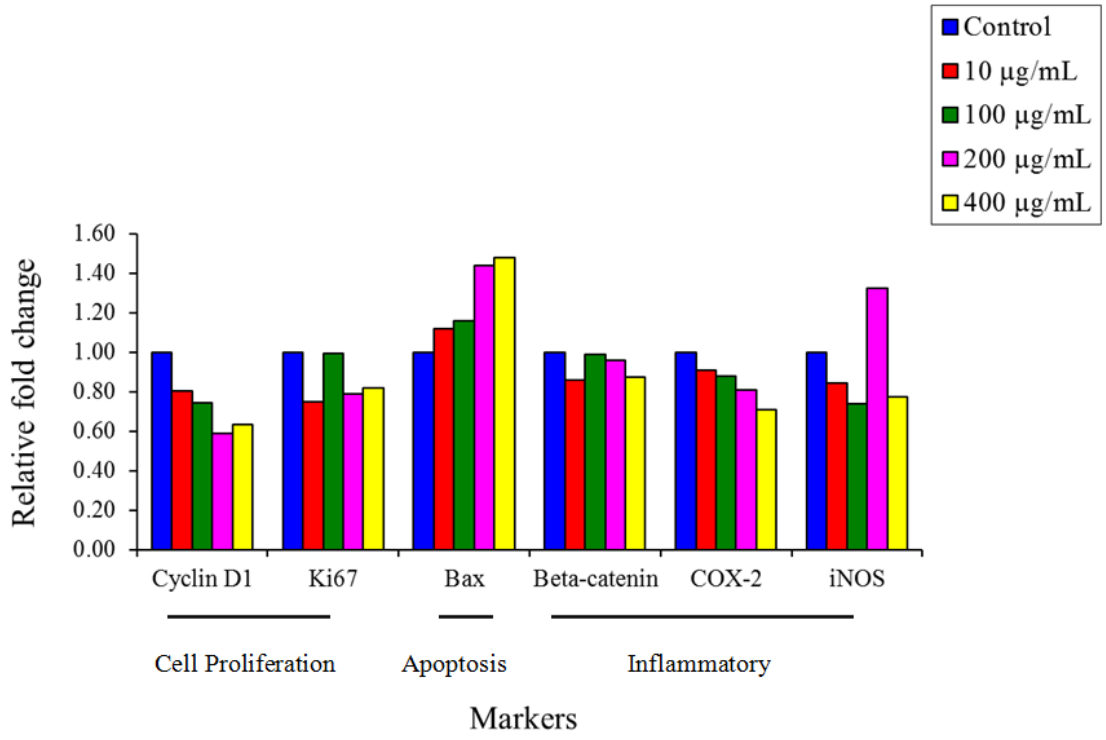


Figure 6.7. Effect of chlorogenic acid on HT-29 cell line.

6.5.3 *p*-Coumaric Acid

In HT-29 cells treated with *p*-coumaric acid, the expression of both the cell proliferation markers, Cyclin D1 and Ki67 was reduced in a concentration dependent manner, with Ki67 showing the highest reduction to 0.29 fold of the control in response to 800 μg/mL (Figure 6.8). In cells treated with at 10 μg/mL, 100 μg/mL p-coumaric acid, the apoptosis marker Bax showed the most significant increase to 2.0-fold, 2.8-fold, while at higher concentrations of 200 μg/mL, 400 μg/mL, and 800 μg/mL p-coumaric acid, the increase was more gradual at 3.0-fold, 3.2-fold, and 3.4-fold, respectively. The three inflammatory markers, β-catenin, COX-2 and iNOS showed no significant change in their expression in response to p-coumaric acid treatment.

6.5.4 Ferulic Acid

Ferulic acid differentially affected the two cell proliferation markers. Cyclin D1 expression was significantly decreased in a concentration-dependent manner, only 10% of the cells were immune-positive when treated with the highest concentration 800 μg/mL (Figure 6.9). Ferulic acid at 10 and 100 μg/mL did not alter Ki67 expression, but at higher concentrations the number of immune-positive cells was gradually reduced at higher concentrations, and at 800 μg/mL only 40% of cells were immunopositive for Ki67. The apoptosis marker Bax increased in a concentration dependent manner, with very high levels of expression (3.5- and 4.5-fold) in cells treated with 400 and 800 μg/mL ferulic acid. The two inflammatory markers, β-catenin and COX-2 did not show much variation in response to ferulic acid treatment. However, iNOS expression reduced to 0.85-fold, 0.67-fold, 0.56-fold, 0.53-fold, and 0.46-fold at 10 μg/mL, 100 μg/mL, 200 μg/mL, 400 μg/mL, and 800 μg/mL of Ferulic acid respectively.

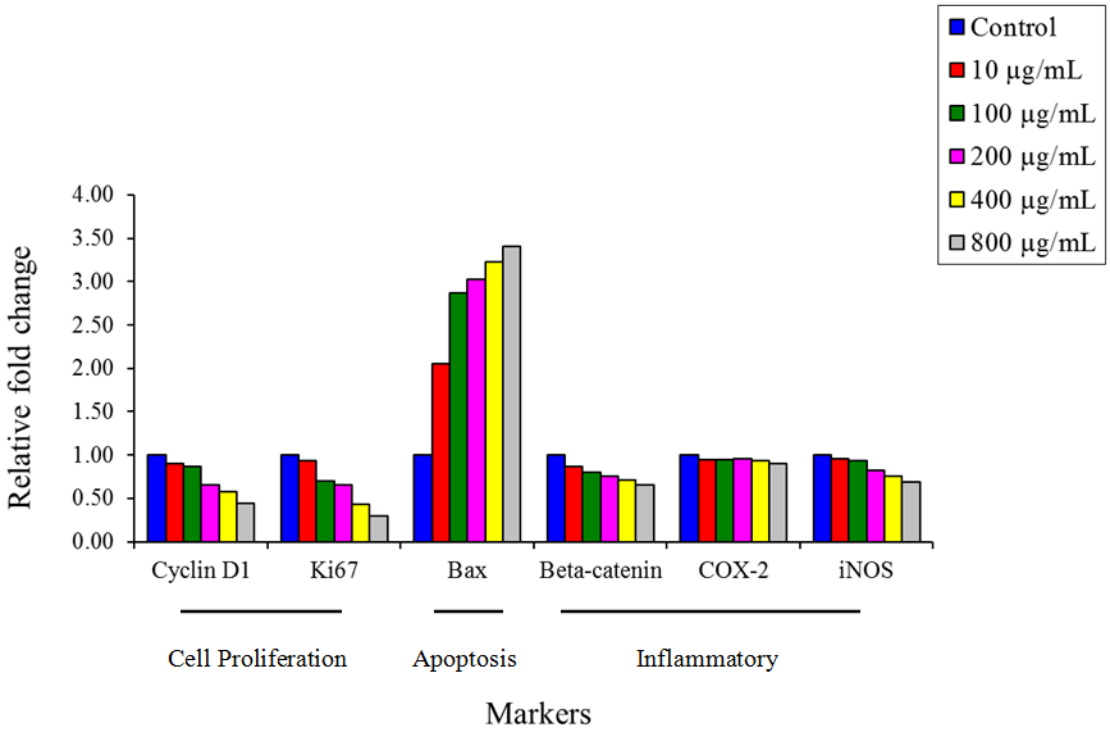


Figure 6.8. Effect of *p*-coumaric acid on HT-29 cell line.

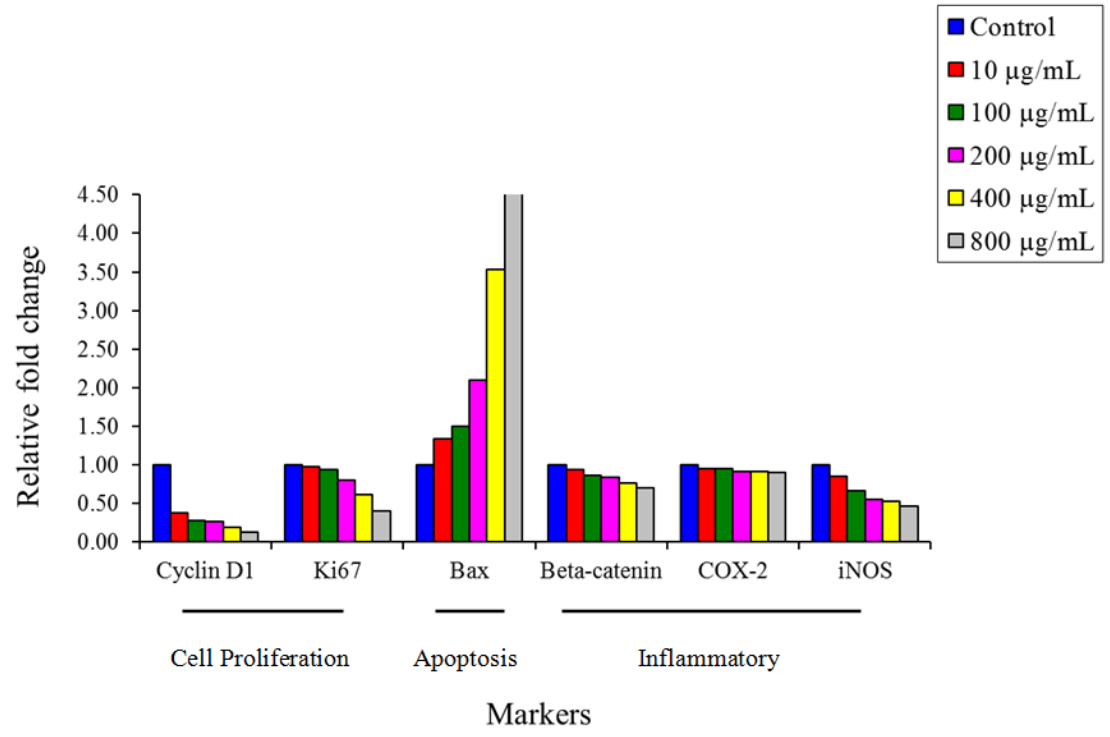


Figure 6.9. Effect of ferulic acid on HT-29 cell line.

6.5.5 Sinapic Acid

The cells treated with sinapic acid (100 and 200 μg/mL) showed a marked increase in Cyclin D1 expression and a slight reduction at other concentrations (Figure 6.10). The other cell proliferation marker Ki67 did not show any specific trends in cells treated with Sinapic acid. In HT-29 cells treated with sinapic acid, at the lowest concentration (10 μg/mL) Bax expression increased to 1.4-fold, but gradually reduced to 0.7-fold, 0.2-fold, 0.5-fold, and 0.7-fold at 100 μg/mL, 200 μg/mL, 400 μg/mL, and 800 μg/mL, respectively (Figure 6.10). Among the three inflammatory markers, β-catenin level reduced to 0.9-fold, 0.8-fold, 0.8-fold, 0.8-fold, and 0.8-fold at 10 μg/mL, 100 μg/mL, 200 μg/mL, 400 μg/mL, and 800 μg/mL of sinapic acid respectively (Figure 6.10). The iNOS expression also reduced to 0.9-fold, 0.8-fold, 0.8-fold, 0.7-fold, and 0.6-fold at 10 μg/mL, 100 μg/mL, 200 μg/mL, 400 μg/mL, and 800 μg/mL of sinapic acid respectively (Figure 6.10). The COX-2 expression did not change much in response to sinapic acid treatment.

6.5.6 3, 4-Dimethoxycinnamic Acid

3, 4-Dimethoxycinnamic acid treated HT-29 cells showed a concentration dependent reduction in the two cell proliferation markers Cyclin D1 and Ki67 (Figure 6.11). At the highest concentration (800 μg/mL), both the Cyclin D1 and Ki67 were reduced to 0.4-fold of the untreated control cells (Figure 6.11). Both the apoptosis marker Bax and the inflammatory markers, β-catenin, COX-2 and iNOS showed no reduction, except Bax and iNOS which were reduced at higher concentrations only (Figure 6.11).

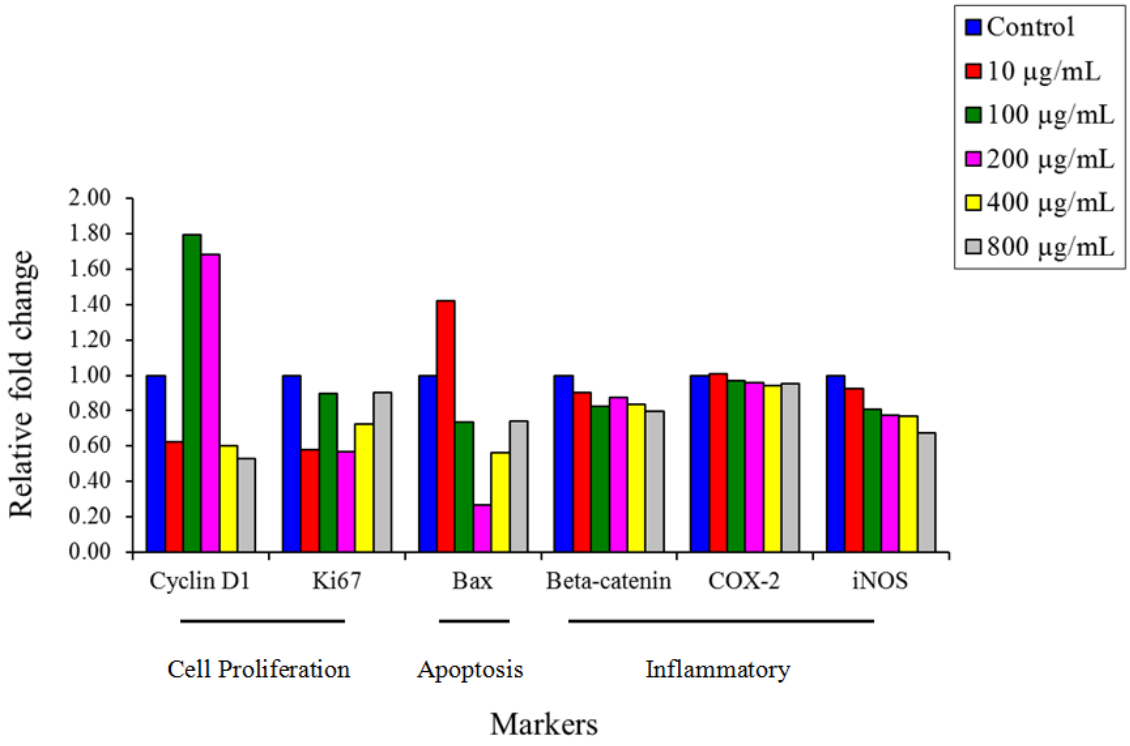


Figure 6.10. Effect of sinapic acid on HT-29 cell line.

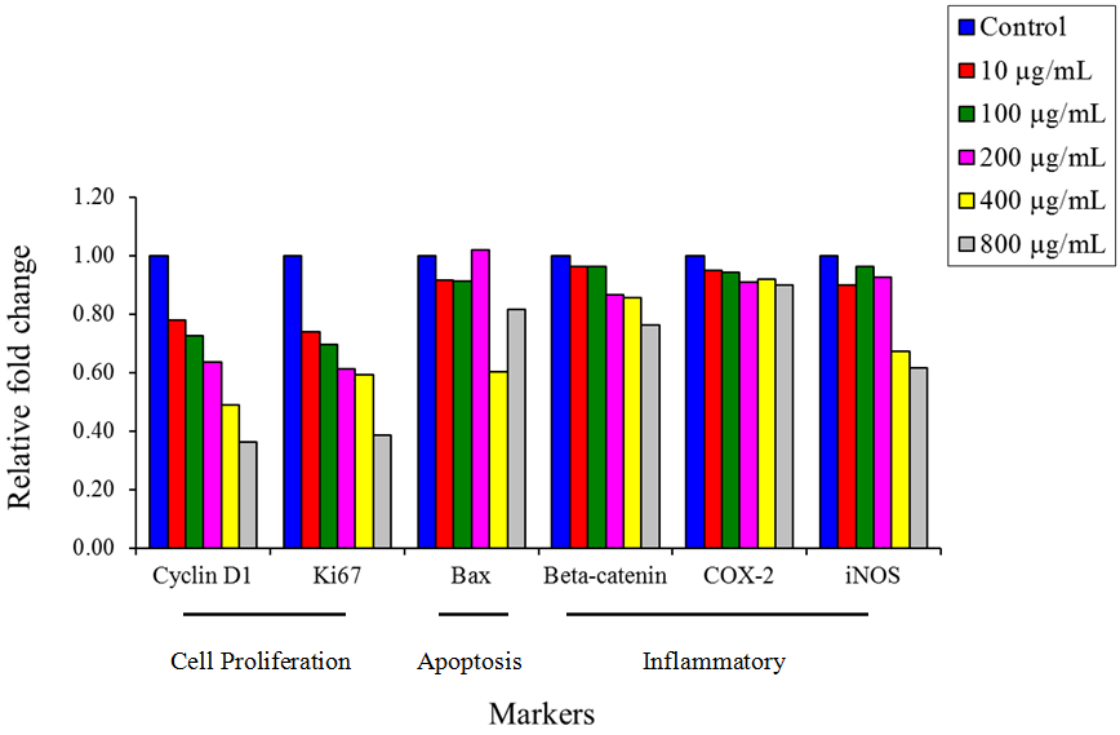


Figure 6.11. Effect of 3, 4-dimethoxycinnamic acid on HT-29 cell line.

6.6 Modulation of Marker Proteins by Benzoic Acid Derivative Phenolic Acids on HT-29 Cell Line

6.6.1 Ellagic Acid

Ellagic acid treatment of HT 29 cells decreased the expression of both the cell proliferation markers Cyclin D1 and Ki67. However, the decrease was more in Cyclin D1 as compared to Ki67 (Figure 6.12). The Bax expression increased in a concentration dependent manner, with the highest concentration (800 μg/mL) showing a 3.1-fold increase over control (Figure 6.12). The three inflammatory markers, β-catenin, iNOS, and COX-2 did not change in response to treatment with Ellagic acid.

6.6.2 Gallic Acid

Gallic acid at higher concentrations was severely detrimental to cell growth. Marker assays were only possible in cells treated with the lowest concentration (10 μg/mL) of Ellagic acid. The cell proliferation marker Cyclin D1 expression was increased to 1.8-fold, while the expression of all other markers either had no effect or showed slight reductions (Figure 6.13).

6.6.3 Protocatechuic Acid

Protocatechuic acid did not show any affect on the cell proliferation markers Cyclin D1 and Ki67, except at the highest concentration (800 μg/mL), where both Ki67 and Cyclin D expression was decreased (Figure 6.14). However, the inhibitory effect at the highest concentration was more pronounced in Cyclin D1 compared to Ki67 (Figure 6.14). The apoptosis marker Bax and inflammatory marker COX-2 did not show any response to treatment of cells with Protocatechuic acid. The other two inflammatory markers β-catenin and iNOS showed slight concentration dependent reduce, but the decrease was less compared to Cyclin D1 (Figure 6.14).

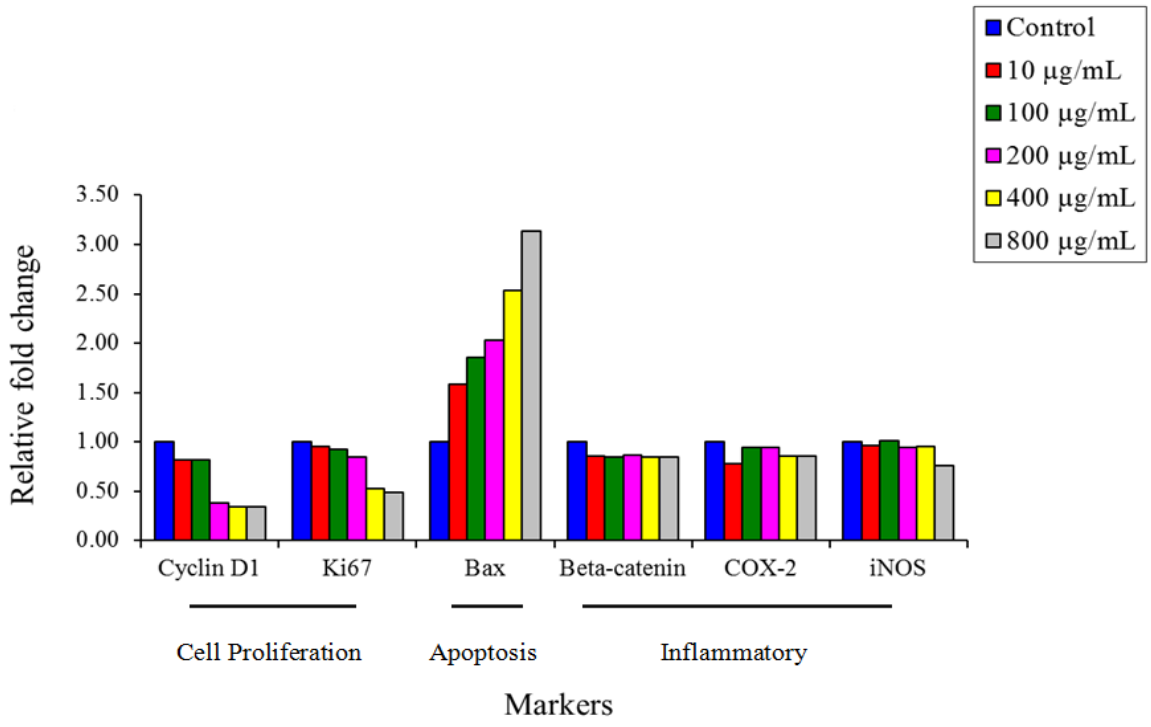


Figure 6.12. Effect of ellagic acid on HT-29 cell line.

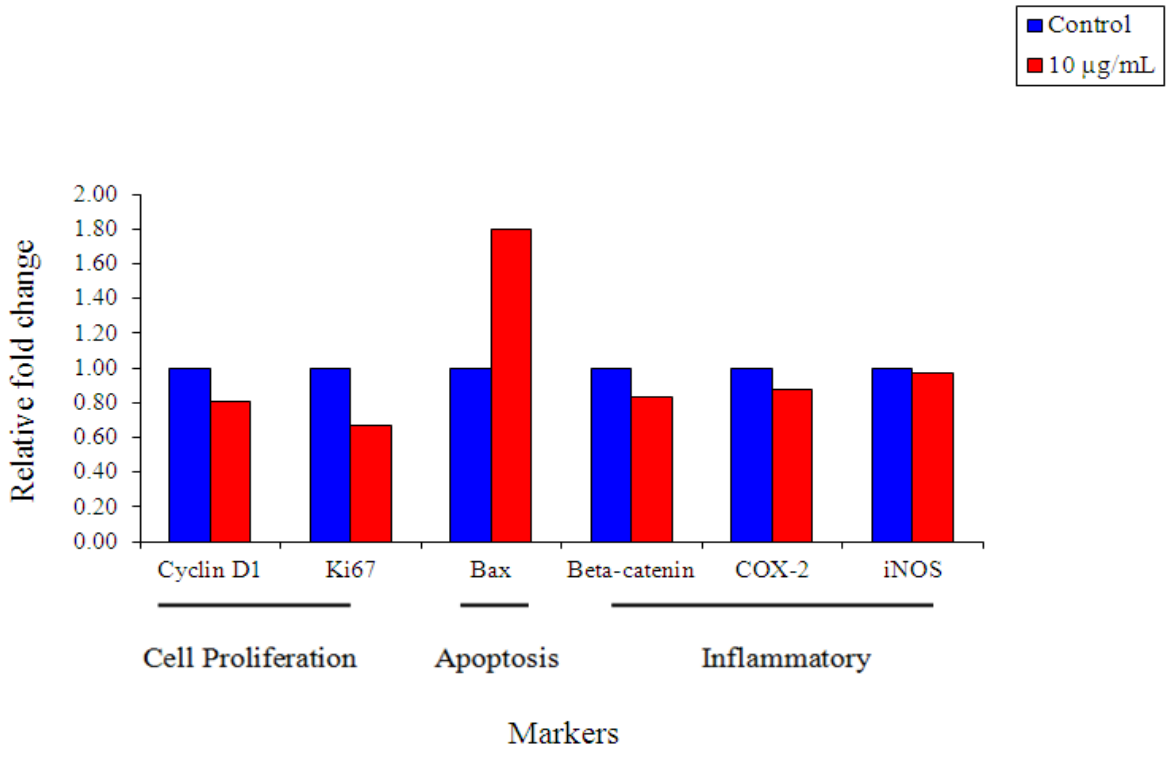


Figure 6.13. Effect of gallic acid on HT-29 cell line.

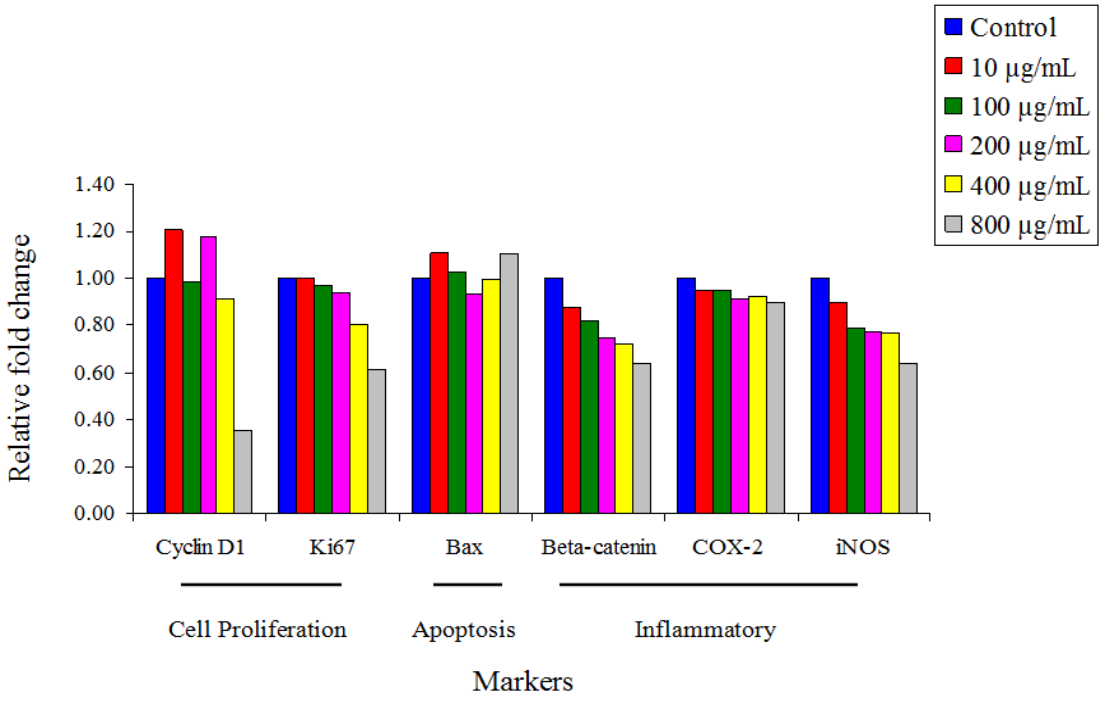


Figure 6.14. Effect of protocatechuic acid on HT-29 cell line.

6.6.4 Salicylic Acid

Treatment of HT-29 cells with salicylic acid decreased the expression of both cell proliferation markers Cyclin D1 and Ki67, however at higher concentrations of salicylic acid Cyclin D1 was reduced more than Ki67 (Figure 6.15). The apoptosis marker Bax expression was significantly increased by almost four fold with the lowest salicylic acid concentration (10 µg/mL). There was some variation with higher concentrations, but the increase in expression was always more than 3.5-fold. Among the inflammatory markers, β-catenin was reduced to 0.7-fold at the lowest salicylic acid concentration (10 µg/mL). The β-catenin levels did not change with increasing salicylic acid concentrations. Salicylic acid did not affect COX-2 and only slightly reduced iNOS levels in HT-29 cells (Figure 6.15).

6.6.5 Syringic Acid

In HT-29 cells, syringic acid treatment in a concentration dependent manner, decreased the expression of cell proliferation marker Cyclin D1, while Ki-67 had no effect except at the highest concentration (800 μg/mL) (Figure 6.16). The apoptosis marker Bax was higher than controls (1.4- to 1.6-fold) at all syringic acid concentrations except that at 200 μg/mL, it was the highest at 2.7-fold more than the control (Figure 6.16). The β-catenin expression was reduced to 0.8-fold at 10 μg/mL of syringic acid, however, increasing syringic acid concentration did not further reduce β-catenin level significantly (Figure 6.16). COX-2 expression was also reduced in a concentration-dependent manner reaching the lowest level of 0.5-fold compared to controls 800 μg/mL of syringic acid (Figure 6.16). The effect of syringic acid on iNOS level did not show clear trends.

6.6.6 Vanillic Acid

Vanillic acid treatment of HT-29 cells produced a differential effect on the two cell proliferation markers analyzed in this study. Cyclin D1 expression was reduced in a concentration-dependent manner, while Ki-67 protein expression was increased by vanillic acid treatment at a concentration as low as 10 μg/mL, when it reached 2.3-fold over non-treated controls (Figure 6.17). At higher vanillic acid concentrations, Ki-67 levels were between 1.6 to 1.9-fold of the non-treated controls (Figure 6.17). The two inflammatory pathway markers, β-catenin and COX-2 did not show much change, while at higher vanillic acid concentrations, iNOS expression showed a slight decrease compared to non-treated controls (Figure 6.17).

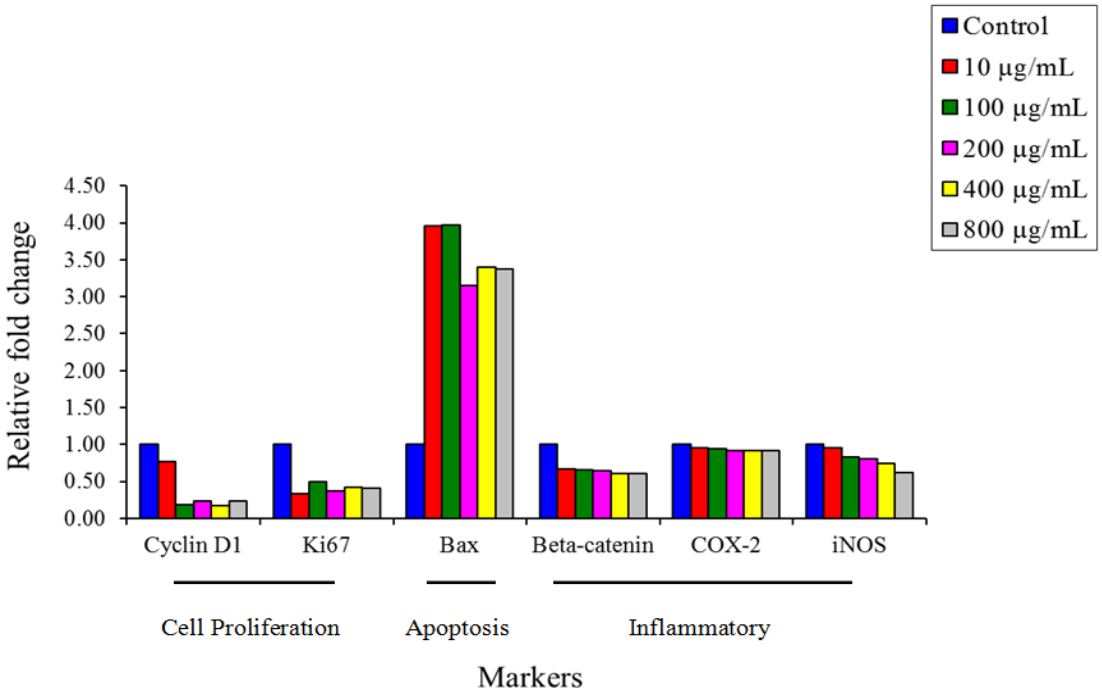


Figure 6.15. Effect of salicylic acid on HT-29 cell line.

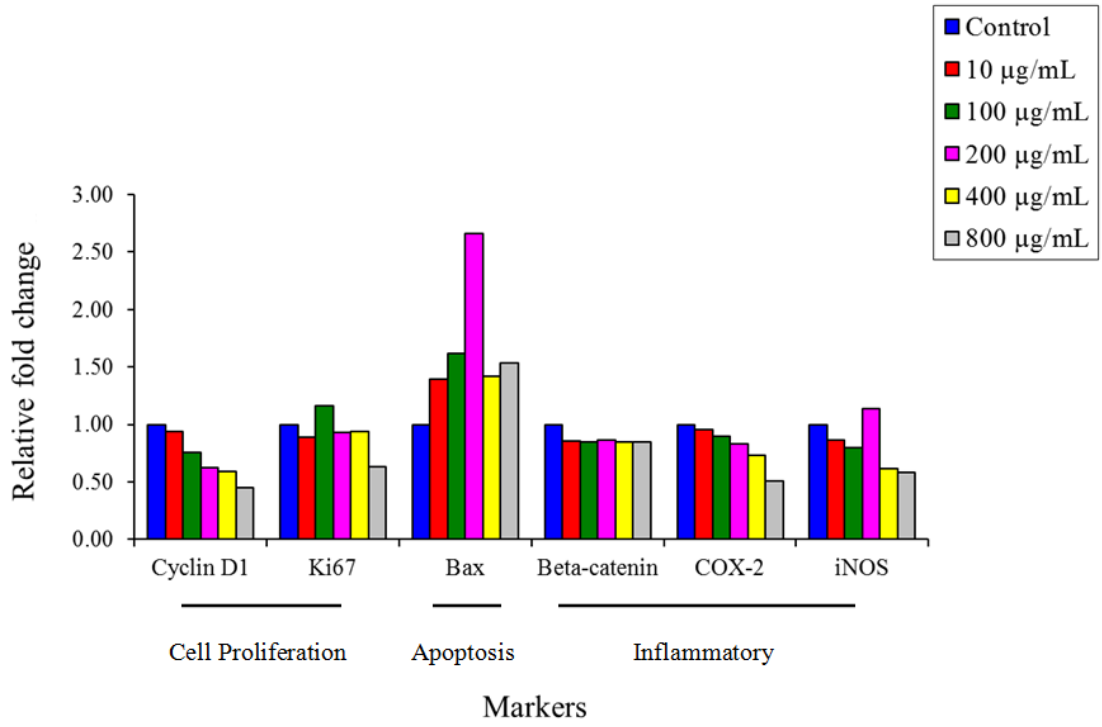


Figure 6.16. Effect of syringic acid on HT-29 cell line.

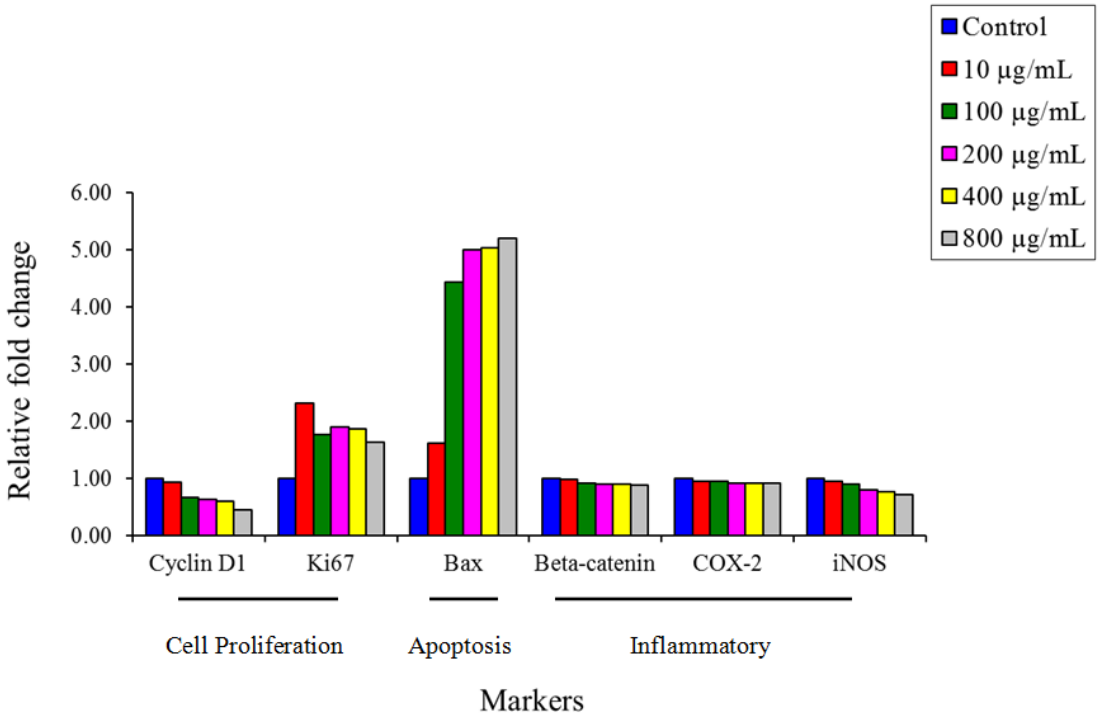


Figure 6.17. Effect of vanillic acid on HT-29 cell line.

6.6.7 2, 5-Dihydroxybenzoic Acid

Treatment of HT-29 cells with 2, 5-dihydrobenzoic acid decreased the expression of all the proteins studied. The expression of cell proliferation markers Cyclin D1 and Ki67 were both reduced, even at the lowest concentrations (10 µg/mL), while at the highest concentration (800 µg/mL) it was approximately 0.1-fold of non-treated control cells (Figure 6.18). The apoptosis marker Bax was not affected by the 2, 5-dihydroxybenzoic lower concentrations (10 and 100 µg/mL), but at higher concentrations (800 µg/mL) it was only 0.4-fold compared to non-treated controls (Figure 6.18). The expression of inflammatory markers was reduced in cells treated with 2, 5-dihydroxybenzoic acid. However, the most drastic decrease was in β-catenin and least

in COX-2 expression. iNOS expression was reduced with increasing the 2, 5-dihydroxybenzoic acid concentration, but the reduction was much less compared to β-catenin (Figure 6.18).

6.6.8 4-Hydroxybenzoic Acid

Expression of both cell proliferation markers Cyclin D1 and Ki67 was reduced in HT-29 cells treated with 4-hydroxybenzoic acid. The most reduction (0.2-fold compared to control) was observed in Ki67 in cells treated with the 4-hydroxybenzoic acid highest concentration (800 µg/mL) (Figure 6.19). The apoptosis marker Bax showed a concentration-dependent increase in cells treated with the 4-hydroxybenzoic acid highest concentration (800 µg/mL) reaching close to seven-fold more than the non-treated controls (Figure 6.19). The three inflammatory markers β-catenin, COX-2 and iNOS did not show much change in response to 4-hydroxybenzoic acid treatment of HT-29 cells.

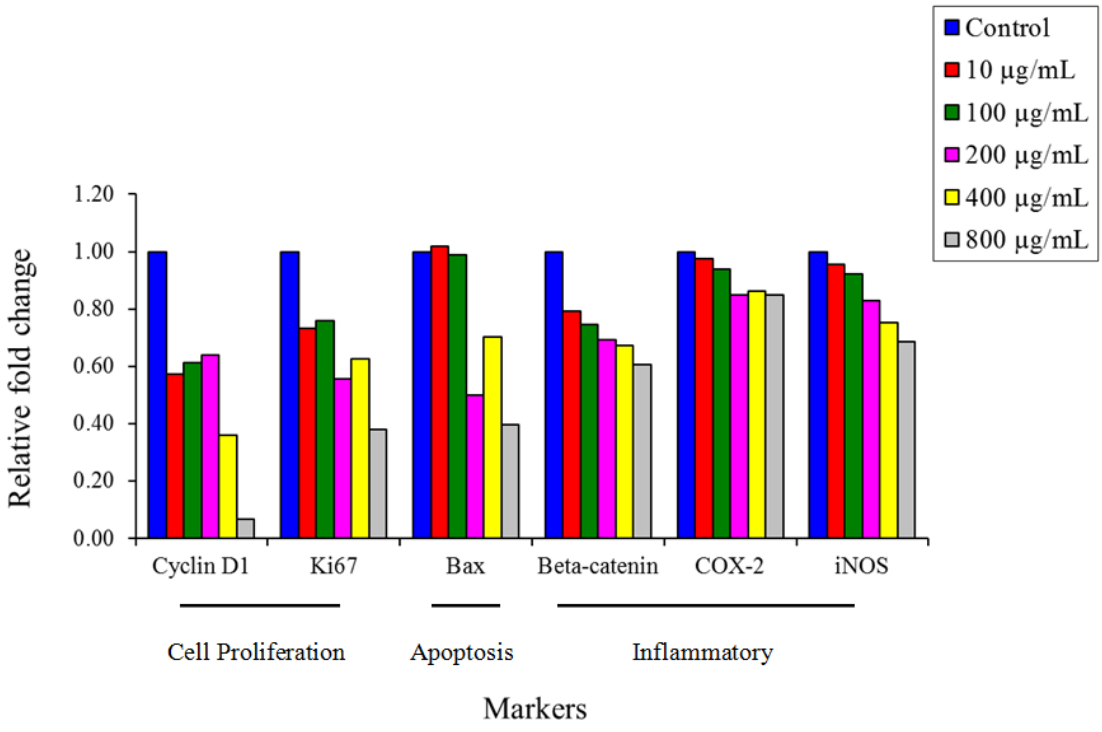


Figure 6.18. Effect of 2, 5-dihydroxybenzoic acid on HT-29 cell line.

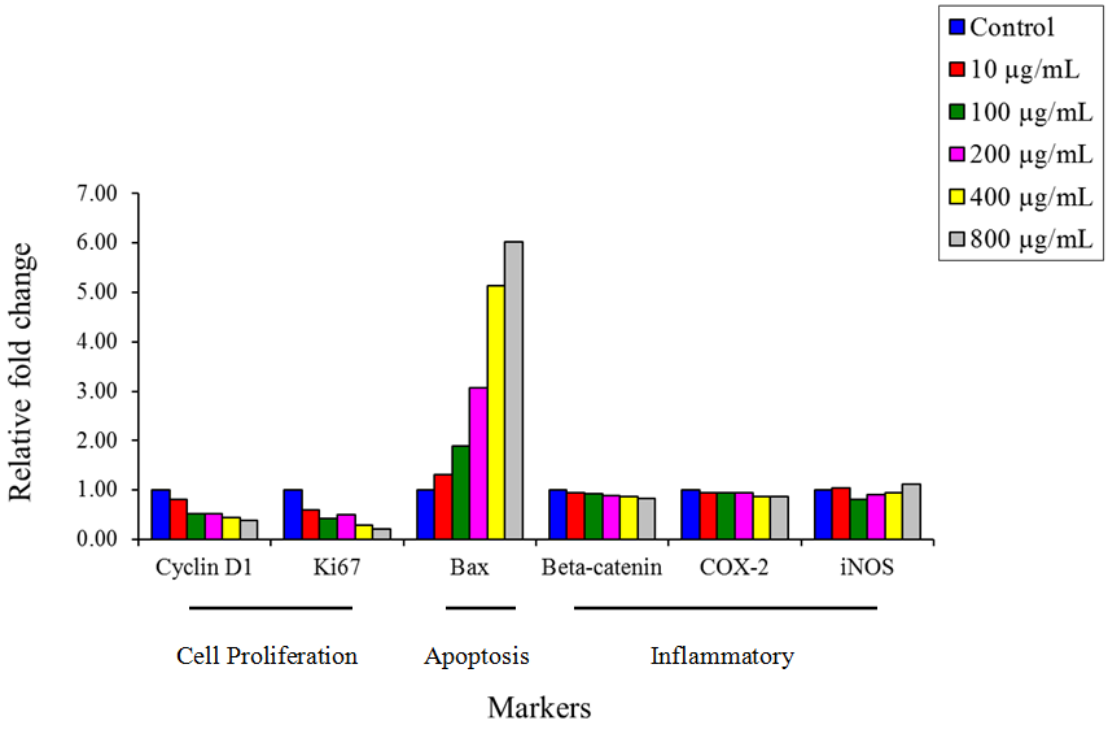


Figure 6.19. Effect of 4-hydroxybenzoic acid on HT-29 cell line.

6.7 Modulation of Marker Proteins by Cinnamic Acid Derivative Phenolic Acids on HCT 116 Cell Line

6.7.1 Caffeic Acid

HT 116 cells were treated with three caffeic acid concentrations (10, 100 and 200 µg/mL) because at higher concentrations, cells were not viable. Among the cell proliferation markers, Cyclin D1 was reduced to half that in non-treated control cells, while Ki67 concentration did not change in response to caffeic acid treatment (Figure 6.20). An apoptosis marker Bax concentration slightly increased with 10 and 100 µg/mL caffeic acid, but when its concentration was increased to 200 µg/mL, Bax protein concentration was increased by five-fold (Figure 6.20). Among the inflammatory markers, β-catenin showed slight reduction, but iNOS was not affected. The Bcl-2 showed a very weak reaction, therefore it was not quantified.

6.7.2 Chlorogenic Acid

In response to chlorogenic acid treatment, a cell proliferation marker, Cyclin D1 expression gradually reduced in a concentration dependent manner, while Ki67 did not show any affect (Figure 6.21). At higher concentrations (200 and 400 μg/mL) of chlorogenic acid, Cyclin D1 expression was reduced to 0.5-fold of the control non treated cells (Figure 6.21). The apoptosis marker Bax at all chlorogenic acid concentrations was more than three-fold higher than of the non-treated control cells (Figure 6.21). The two inflammatory markers studied, β-catenin showed some reduction in response to chlorogenic acid, but iNOS concentration was similar in both treated and non-treated control cells.

6.7.3 *p*-Coumaric Acid

The effect of *p*-coumaric acid on the presence of two cell proliferation markers is inconsistent (Figure 6.22). Cyclin D1 was slightly reduced at lower concentrations of *p*-coumaric acid, but at its higher concentrations it was almost the same as in non-treated control cells (Figure 6.22). Ki67 did not show much reduction at all concentrations of *p*-coumaric acid except at its highest concentration, Ki67 expression was almost half of that in non-treated control cells (Figure 6.22). The apoptosis marker Bax was reduced to half of the non-treated control cells at the lowest concentration (10 μg/mL), but it increased to 2.0- to 4.5- fold at all other concentrations of *p*-coumaric acid (Figure 6.22). Among the inflammatory markers, β-catenin showed slight reduction but iNOS did not change in response to *p*-coumaric acid treatment.

6.7.4 Ferulic Acid

Ferulic acid treatment at the highest concentration (800 μg/mL) caused some reduction in the cell proliferation markers Cyclin D1 and Ki67 which were reduced to almost half the

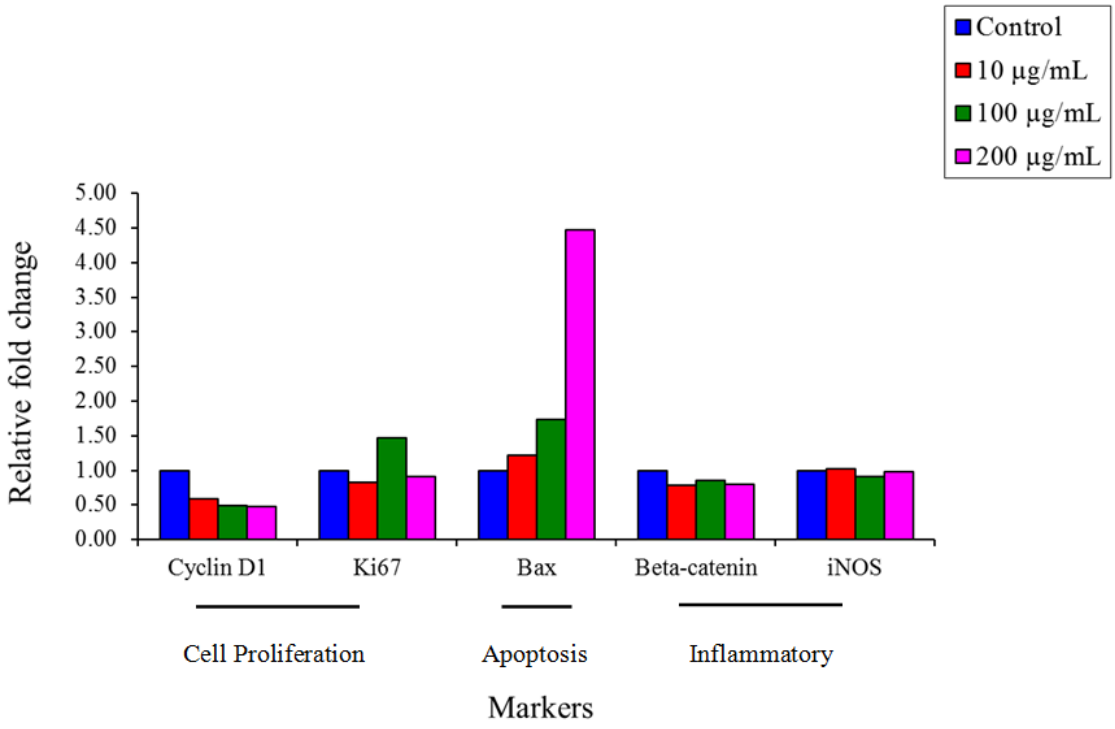


Figure 6.20. Effect of caffeic acid on HCT 116 cell line.

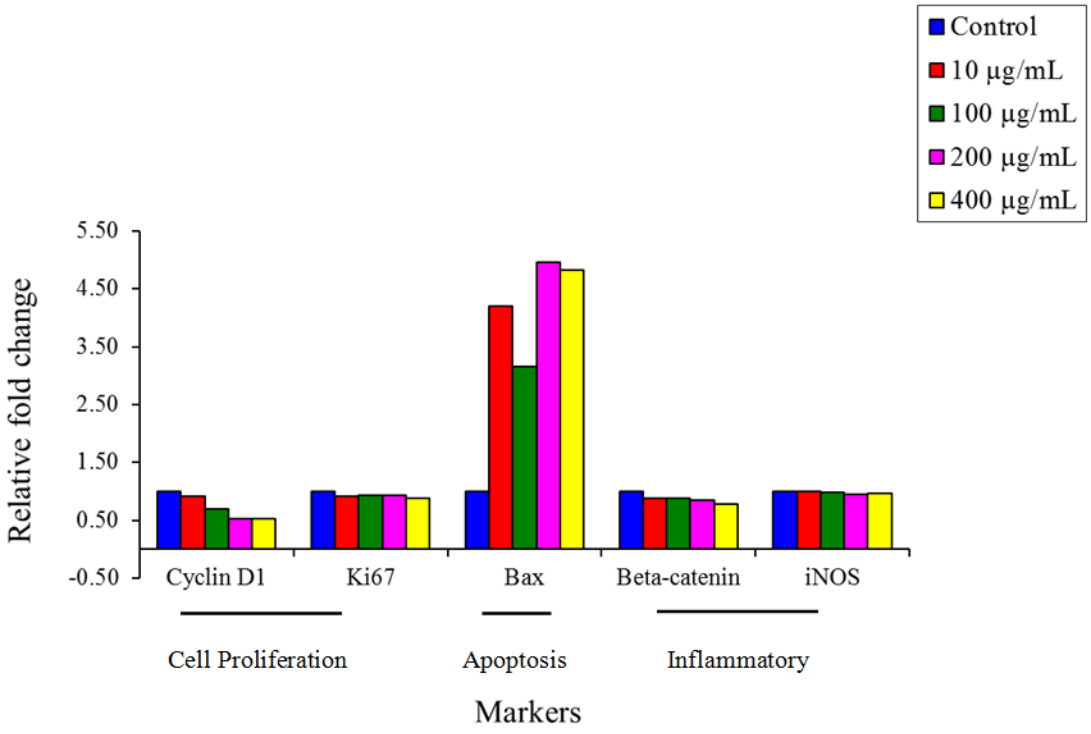


Figure 6.21. Effect of chlorogenic acid on HCT 116 cell line.

concentration present in non-treated control cells (Figure 6.23). The apoptosis marker Bax increased 1.5-, 1.9-, 2.8-, 2.0-, and 3.1-fold at 10 μg/mL, 100 μg/mL, 200 μg/mL, 400 μg/mL, and 800 μg/mL of ferulic acid, respectively, compared to non-treated control cells (Figure 6.23). Inflammatory marker β-catenin concentration reduced slightly at lower concentrations of ferulic acid treatment but at highest concentration (800 μg/mL) it was only 0.4-fold of non-treated control cells of ferulic acid (Figure 6.23). However, iNOS, another inflammatory marker studied did not change in its concentration in response to ferulic acid treatment.

6.7.5 Sinapic Acid

Sinapic acid treatment reduced both the cell proliferation markers Cyclin D1 and Ki67. Cyclin D1 concentration decreased gradually and directly in relation to the amount of sinapic acid, while Ki67 did not change at most of the sinapic acid concentrations (Figure 6.24). At 800 μg/mL sinapic acid both Cyclin D1 and Ki67 were only 0.2-fold of the non-treated control cells. The apoptosis marker Bax did not change at10 μg/mL, sinapic acid, but at its higher concentrations Bax concentration was 3.0- to 4.8-fold higher than the non-treated control cells (Figure 6.24). The two inflammatory markers β-catenin and iNOS did not show consistent variation dependent upon Sinapic acid concentration compared to non-treated control cells.

6.7.6 3, 4-Dimethoxycinnamic Acid

Two cell proliferation markers behaved differently in response to 3, 4-dimethoxycinnamic acid treatment. The lowest 3, 4-dimethoxycinnamic acid concentration slightly reduced Cyclin D1 concentration, but its expression remained the same as in non-treated control cells when cells were treated with increasing 3, 4-dimethoxycinnamic acid concentrations (Figure 6.25). Contrary to Cyclin D1, Ki67 expression was not affected at lower 3, 4-dimethoxycinnamic acid concentrations, at 400 and 800 μg/mL, Ki67 expression was almost

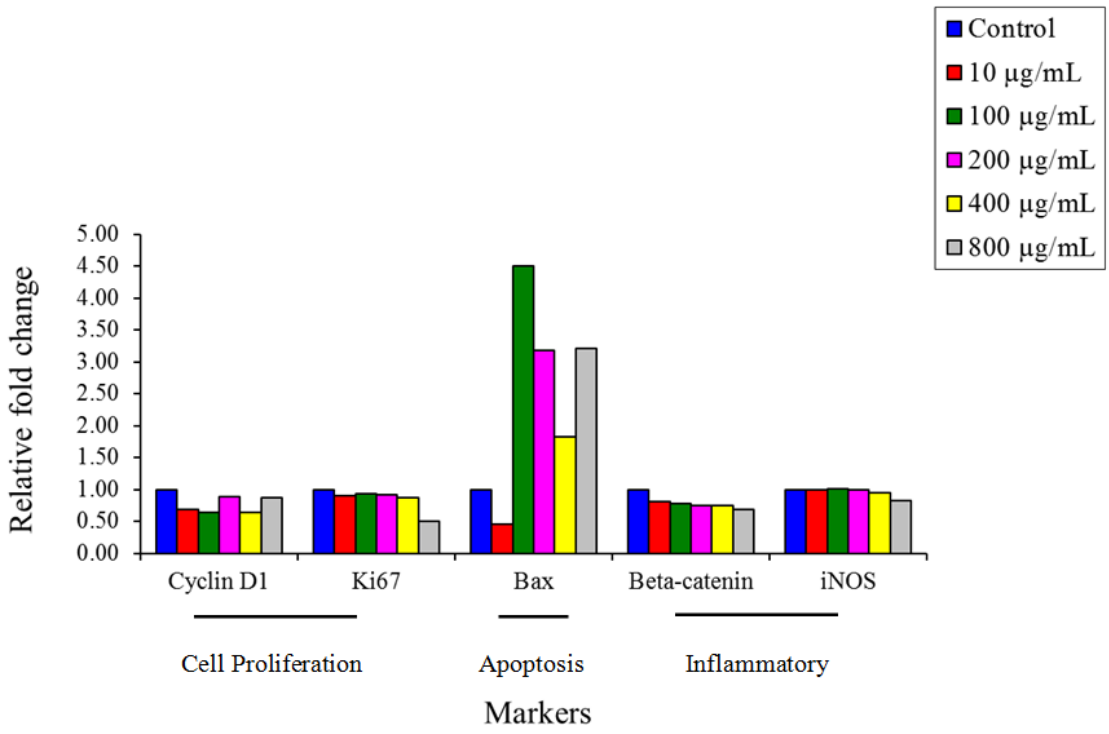


Figure 6.22. Effect of *p*-coumaric acid on HCT 116 cell line.

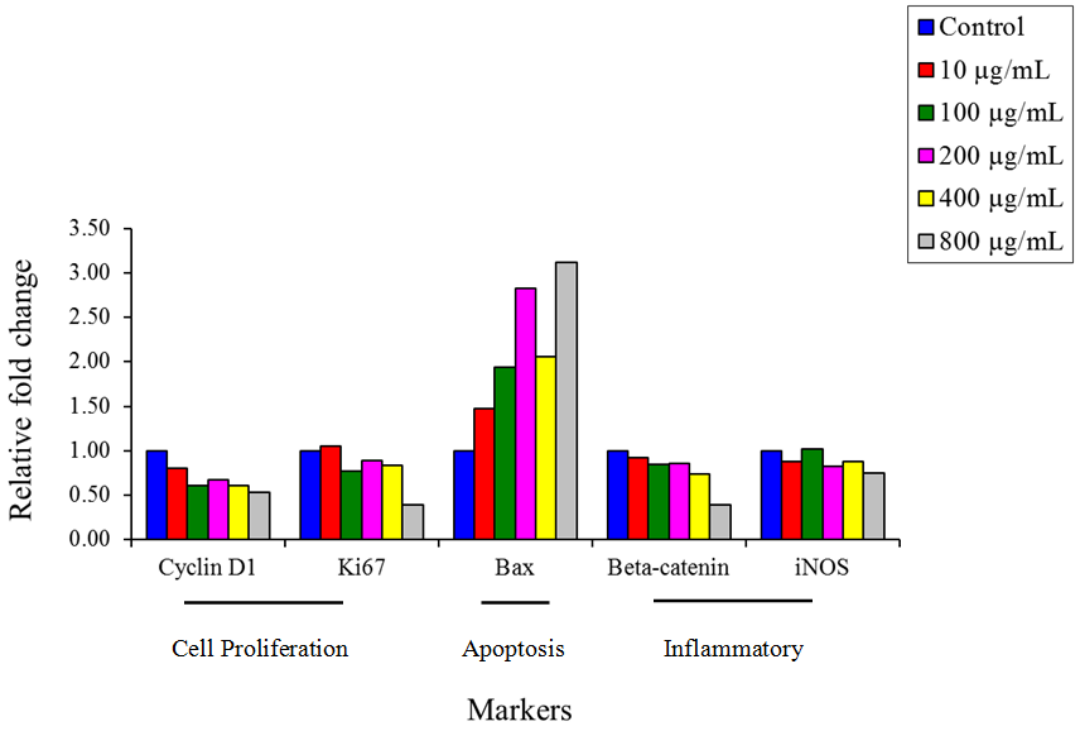


Figure 6.23. Effect of ferulic acid on HCT 116 cell line.

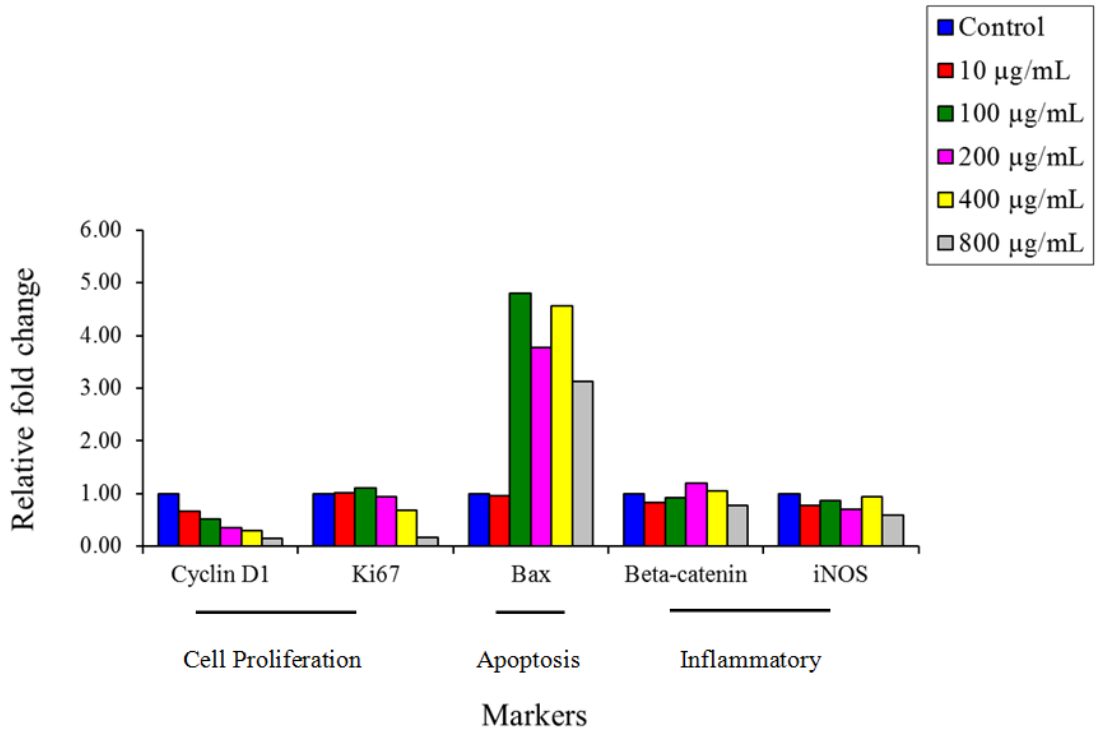


Figure 6.24. Effect of sinapic acid on HCT 116 cell line.

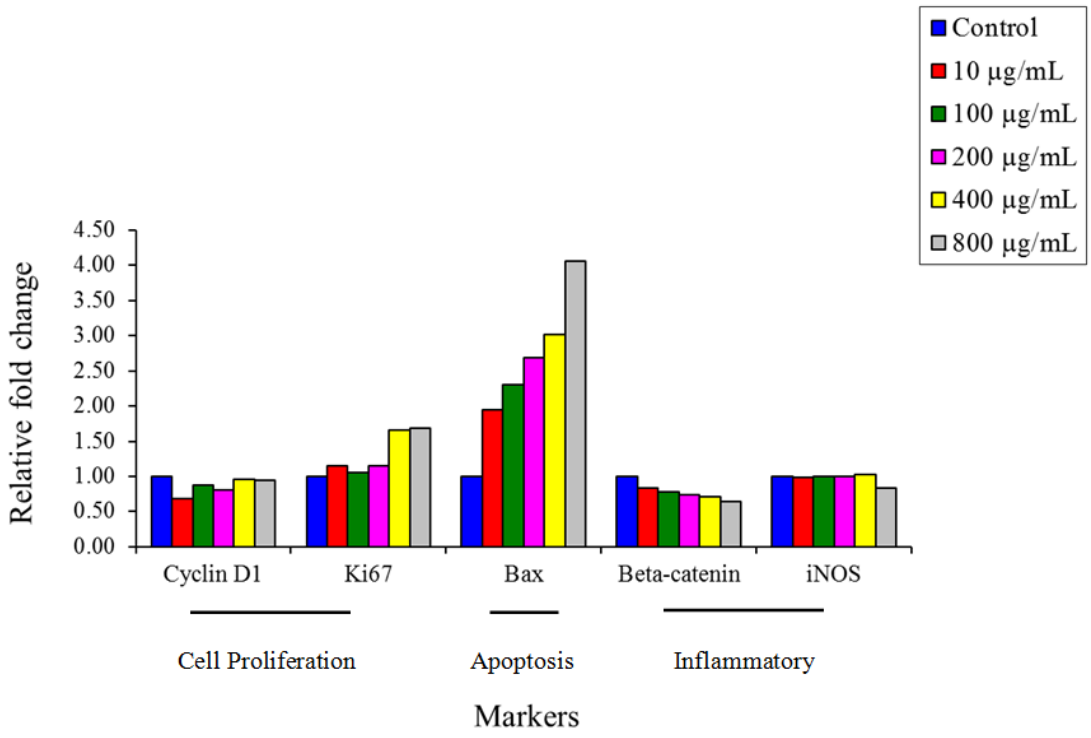


Figure 6.25. Effect of 3, 4-dimethoxycinnamic acid on HCT 116 cell line.

two-fold higher than un-treated control cells (Figure 6.25). The apoptosis marker Bax at the lowest concentration (10 μg/mL) increased to almost 2-fold and with increasing concentration gradually increased reaching to four-fold higher compared to non-treated control cells (Figure 6.25). Increasing 3, 4-dimethoxycinnamic acid concentration, gradually reduced β-catenin, by 0.8- to 0.6-fold compared to non-treated control cells. iNOS levels were similar to non-treated control cells at most of 3, 4-dimethoxycinnamic acid concentrations tested.

6.8 Modulation of Marker Proteins by Benzoic Acid Derivative Phenolic Acids on HCT 116 Cell Line

6.8.1 Ellagic Acid

Both the cell proliferation markers were reduced in HCT 116 cells treated with ellagic acid. Cyclin D1 was reduced to 0.4-fold even at the lowest concentration (10 μg/mL) of ellagic acid, and its increasing concentration gradually reduced Cyclin D1 to almost 10% compared to non-treated control cells (Figure 6.26). Ellagic acid slightly reduced Ki67 expression, except its highest (800 μg/mL) concentration, reduced Ki67 to almost 0.4-fold compared to non-treated control cells. Increasing ellagic acid concentrations, gradually increased apoptosis marker Bax from 2.0- to 4.5-fold compared to non-treated control cells (Figure 6.26). Among the two inflammatory markers, increasing ellagic acid concentrations, gradually reduced β-catenin from 0.9- to 0.5-fold compared to non-treated control cells. At lower concentrations (10, 100 and 200 μg/mL) of ellagic acid, iNOS was slightly reduced, however ellagic acid (400 μg/mL, reduced iNOS to 0.5-fold compared to non-treated control cells (Figure 6.26).

6.8.2 Gallic Acid

Gallic acid was highly toxic to HCT 116 cells, and sufficient numbers of cells were obtained from samples treated with the lowest concentration (10 μg/mL) (Figure 6.27). All the markers were reduced except the apoptosis marker Bax, which showed a slight increase to 1.2-fold of the non-treated control cells (Figure 6.27). The inflammatory marker iNOS did not show any change in response to gallic acid treatment.

6.8.3 Protocatechuic Acid

All the cell proliferation and inflammatory markers were present in similar amounts in all concentrations of protocatechuic acid treated cells or non-treated control cells (Figure 6.28). Apoptosis marker Bax at lowest concentration increased slightly, but at higher concentrations (100 – 800 μg/mL) varied between 1.9- to 2.5-fold as compared to non-treated control cells (Figure 6.28).

6.8.4 Salicylic Acid

The two cell proliferation markers studied behaved differently in response to salicylic acid treatment. Increasing concentrations of salicylic acid gradually reduced Cyclin D1 amount, with the highest salicylic acid concentration reducing Cyclin D1 to 0.64-fold compared to non-treated control cells (Figure 6.29). Ki67 marker increased in response to salicylic acid treatment, albeit very little at lower concentrations, but at higher concentrations (400 and 800 μg/mL) it increased to 1.7-fold compared to non-treated control cells (Figure 6.29). The apoptosis marker Bax and inflammatory markers β-catenin and iNOS, either did not change or were only slightly reduced in amount compared to non-treated control cells (Figure 6.29).

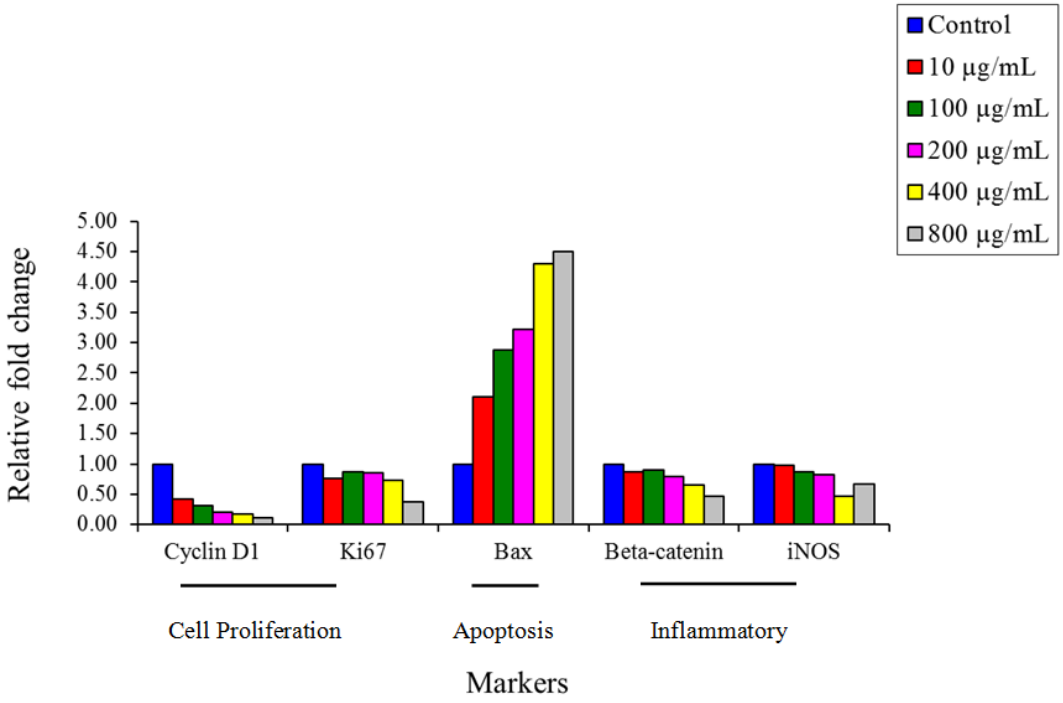


Figure 6.26. Effect of ellagic acid on HCT 116 cell line.

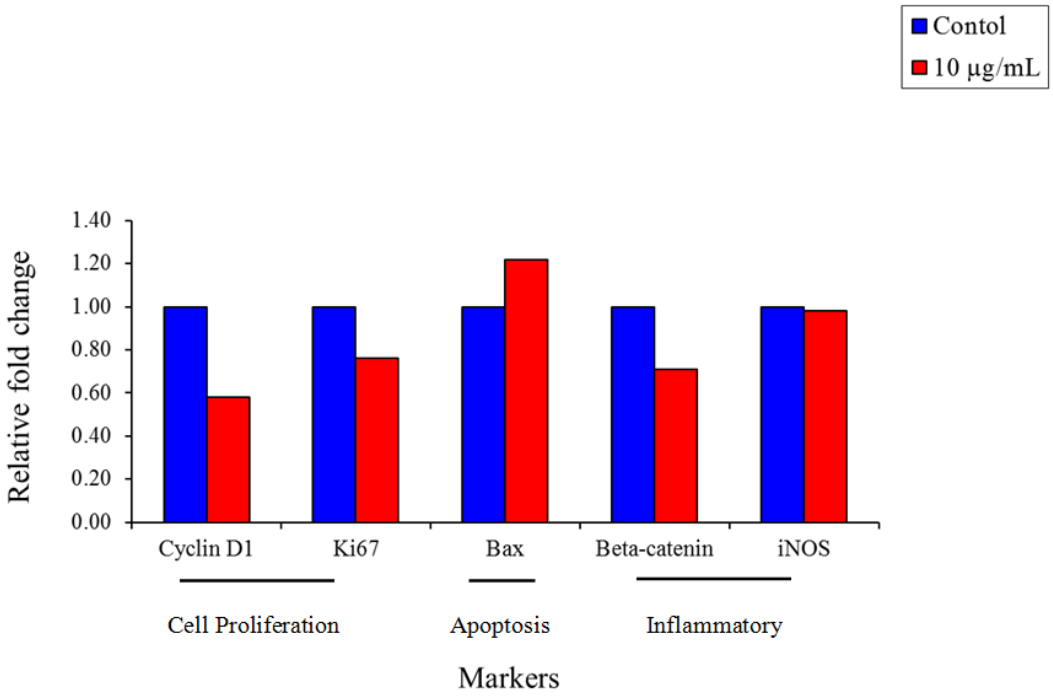


Figure 6.27. Effect of gallic acid on HCT 116 cell line.

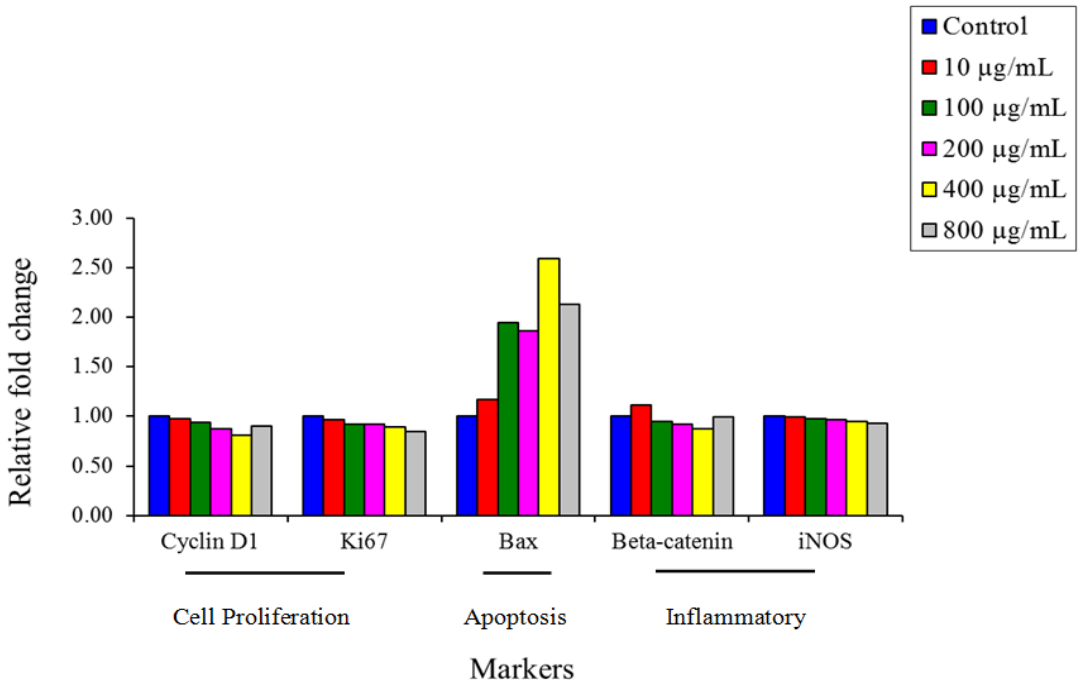


Figure 6.28. Effect of protocatechuic acid on HCT 116 cell line.

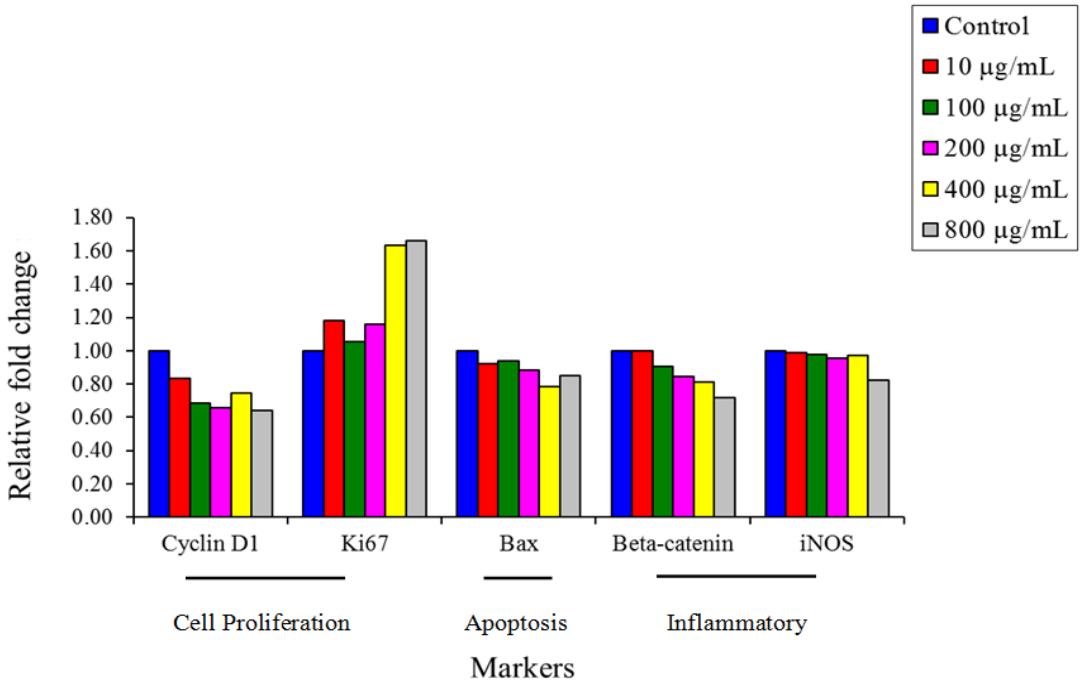


Figure 6.29. Effect of salicylic acid on HCT 116 cell line.

6.8.5 Syringic Acid

Syringic acid, in a concentration-dependent manner, reduced the cell proliferation marker Cyclin D1 expression compared to non-treated controls (Figure 6.30). On the other hand, lower concentrations of syringic acid did not affect Ki67 amounts, but at higher concentrations (400 and 800 μg/mL), Ki67 expression was doubled as compared to non-treated control cells (Figure 6.30). Syringic acid in a concentration dependent manner increased an apoptosis marker Bax from 2.55-fold at lowest concentration (10 μg/mL) of syringic acid and up to 3.6-fold at higher concentrations (Figure 6.30). The inflammatory markers, β-catenin and iNOS showed minor variations in response to syringic acid treatment.

6.8.6 Vanillic Acid

Vanillic acid treatment of cells showed a concentration dependent decrease in Cyclin D1, which at the highest vanillic acid concentration, was reduced to 0.4-fold of non treated cells (Figure 6.31). The amount of another cell proliferation marker Ki67 did not show any definite trends of reduced amounts in response to vanillic acid treatment (Figure 6.31). Vanillic acid treatment of HCT 116 cells induced large amounts of apoptosis marker Bax, which varied from 4- to 9-fold higher compared to non-treated cells (Figure 6.31). The inflammatory markers β-catenin and iNOS did not show any specific trends in response to vanillic acid treatment (Figure 6.31).

6.8.7 2, 5-Dihydroxybenzoic acid

Increasing concentrations of 2, 5-dihydroxybenzoic acid treatment of cells reduced a cell proliferation marker Cyclin D1 to a low of 0.5-fold compared to non-treated cells (Figure 6.32). However, another cell proliferation marker Ki67 did not change much at lower concentrations of 2, 5-dihydroxybenzoic acid treatment, but at its highest concentration, Ki67 was reduced to

almost 0.3-fold of non-treated cells (Figure 6.32). 2, 5-Dihydroxybenzoic acid treatment of HCT 116 cells increased an apoptosis marker Bax. Increasing concentrations, of 2, 5-dihydroxybenzoic acid, up to 200 μg/mL, Bax gradually increased to 5-fold compared to non-treated cells (Figure 6.32). However, at higher concentrations (400 and 800 μg/mL) of 2, 5-dihydroxybenzoic acid, Bax was reduced to 1.8- and 1.2-fold compared to non treated cells (Figure 6.32). Among the two inflammatory markers, β-catenin did not show any change in response to 2, 5-dihydroxybenzoic acid treatment, while iNOS showed some reduction compared to non-treated cells.

6.8.8 4-Hydroxybenzoic acid

HCT 116 cells treated with 4-hydroxybenzoic acid reduced the amount of cell proliferation marker Cyclin D1, which at the highest concentration (800 μg/mL, 4-hydroxybenzoic acid) was reduced to 0.3-fold compared to non treated cells (Figure 6.33). However, another cell proliferation marker Ki67 did not change or show much reduction in amount compared to non-treated cells (Figure 6.33). 4-Hydroxybenzoic acid treatment of cells increased an apoptosis marker Bax compared to non treated cells, except in cells treated with the highest (800 μg/mL) concentration (Figure 6.33), where it was in similar quantities as in non treated cells (Figure 6.33). In HCT 116 cells, 4-hydroxy benzoic acid treatment, inflammatory markers β-catenin and iNOS did not show much change compared to non treated cells, except high concentrations (400 and 800 μg/mL), iNOS was reduced to 60% of non treated cells (Figure 6.33).

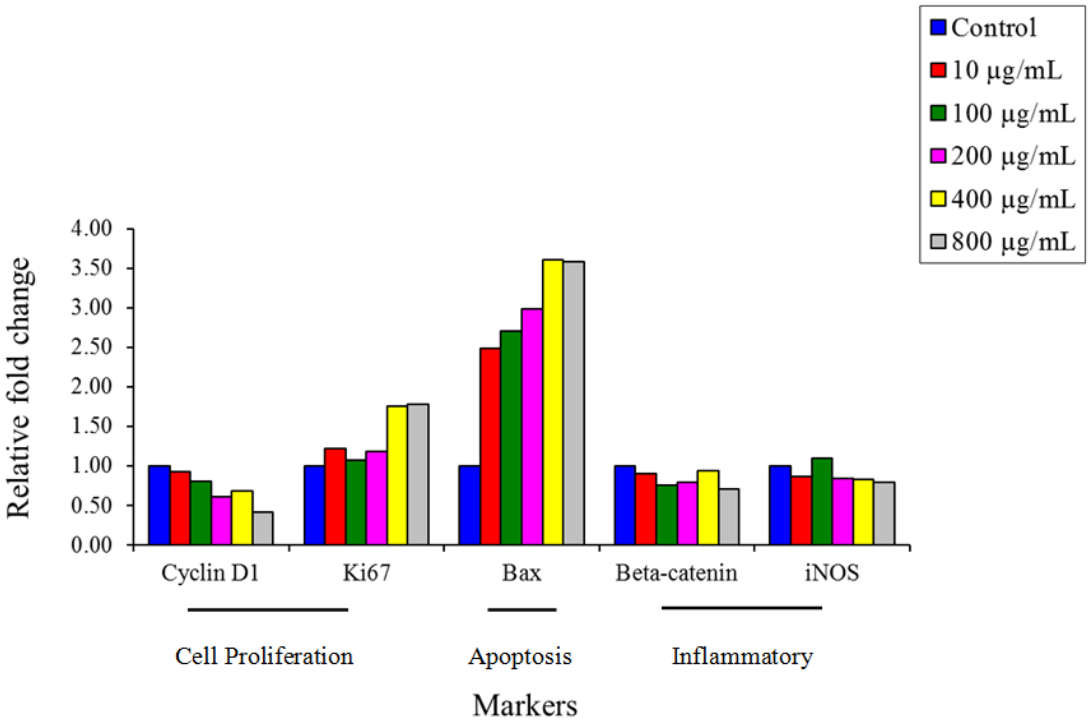


Figure 6.30. Effect of syringic acid on HCT 116 cell line.

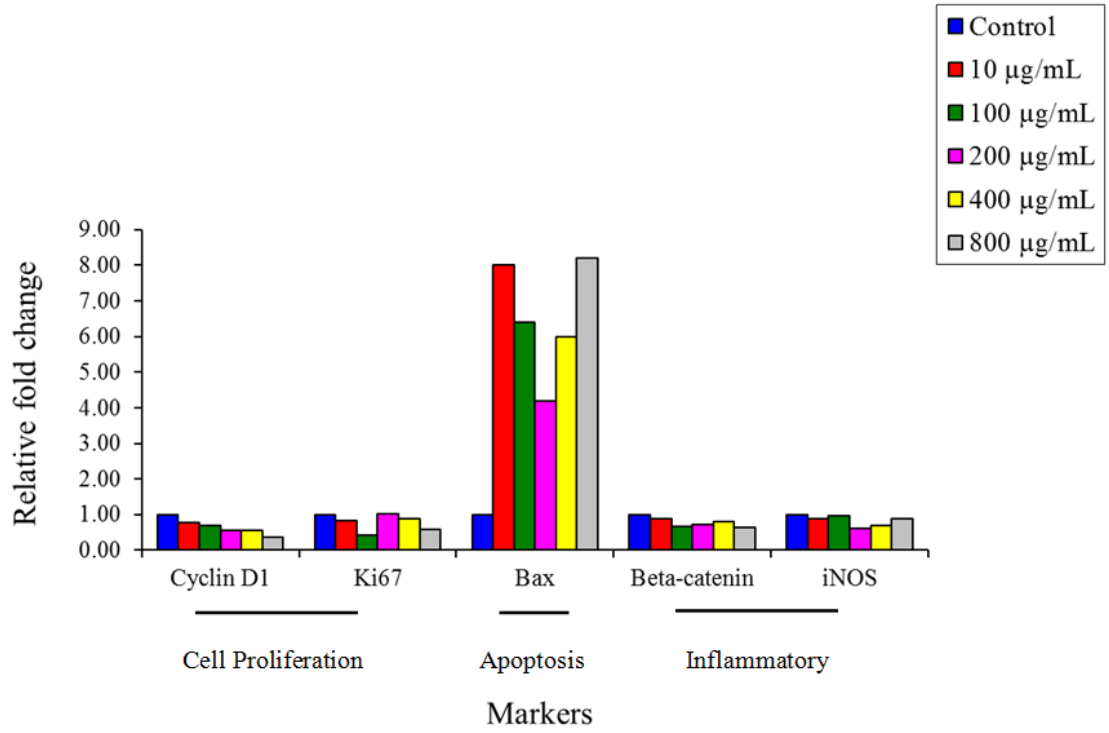


Figure 6.31. Effect of vanillic acid on HCT 116 cell line.

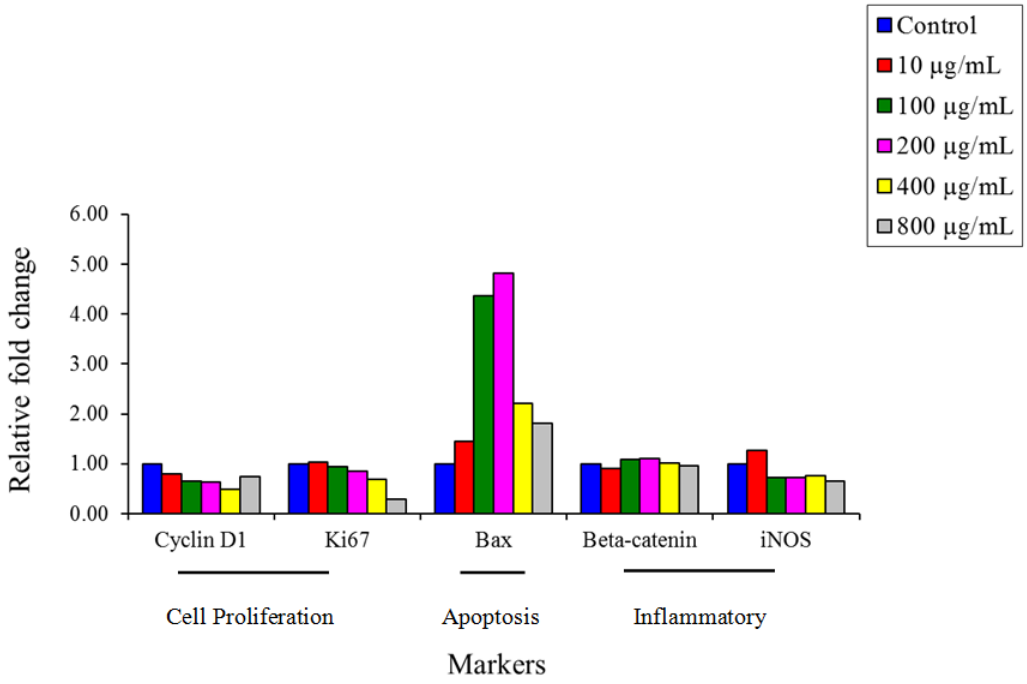


Figure 6.32. Effect of 2, 5-dihydroxybenzoic acid on HCT 116 cell line.

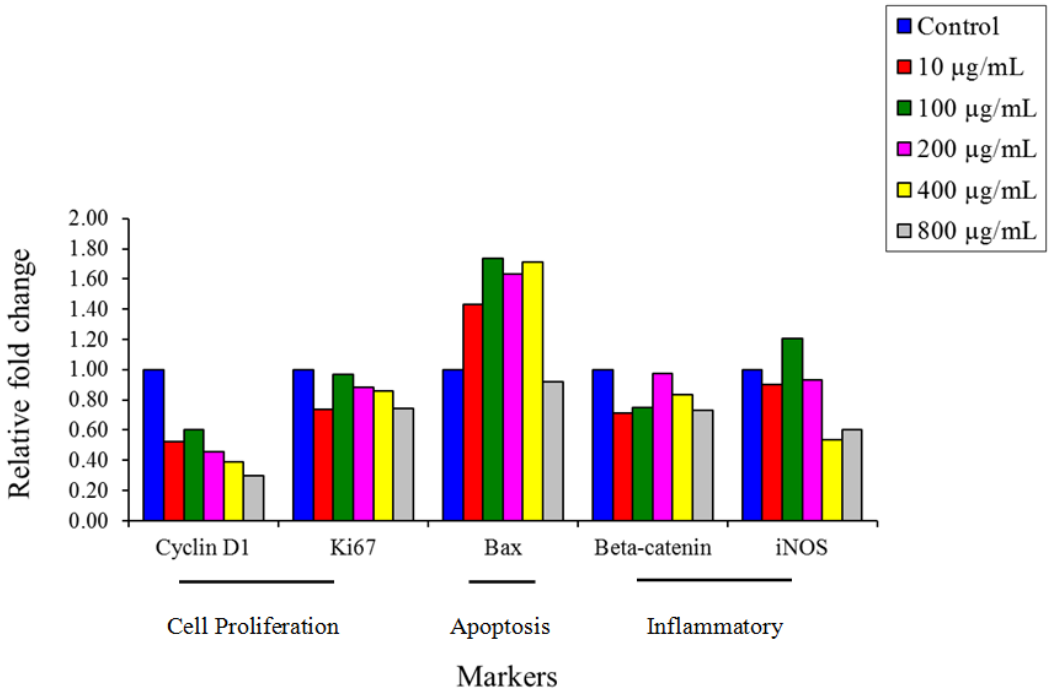


Figure 6.33. Effect of 4-hydroxybenzoic acid on HCT 116 cell line.

6.9 Western Blot Analysis

To confirm the results of immunohistochemical studies, we performed Western Blot analysis using crude cell extracts prepared from cells treated with selected individual phenolic acids. Caffeic, ellagic, and gallic acids were used for confirmatory assays using HCT 116 cell line, while caffeic, gallic, and chlorogenic acid was used with HT-29 cell line. Apoptosis marker Bax and cell proliferation marker Cyclin D1, both of which showed the highest degree of variation in immunohistochemical assays were used to confirm the results using western blot analyses. Polyclonal antibodies against β-actin were used as internal standard to normalise the amount of proteins loaded on to SDS-PAGE gels for analyses.

All the antibodies reacted with respective polypeptides (Figures 6.34 – 6.37). Significant variation in reaction was observed with polypeptide specific antibodies, but as expected β-actin antibodies showed very similar reaction with all cell extracts in respective treatment. This suggests that similar amounts of protein were loaded on the gels in respective treatments. A quantitative estimation was made by quantifying the band intensity and comparing the intensity with non-treated control cells (Appendix 13). In HCT 116 cells, caffeic and ellagic acid treatments showed an increase in Bax polypeptide, with highest increase of 3.5-fold was observed with 200 µg/mL caffeic acid (Figure 6.38). Gallic acid was highly toxic to cells and viable cells were obtained only at 10 µg/mL, and at this concentration Bax polypeptide band intensity did not differ from untreated control cells. Cyclin D1 band intensity was decreased in all treatments, with most decrease observed 400 µg/mL ellagic acid (Figure 6.38).

In HT-29 cells, caffeic acid and chlorogenic acid increased the band intensity of Bax polypeptides. The highest concentration (800 µg/mL) of chlorogenic acid increased Bax polypeptide intensity by more than three-fold (Figure 6.39). Viable cells were obtained with

only 10 µg/mL gallic acid, at which the Bax polypeptide intensity did not differ from untreated control cells. Caffeic acid at the lowest concentration (10 µg/mL) drastically reduced the intensity of Cyclin D1 immunoreactive band. Both chlorogenic acid and gallic acid treatment showed some reduction in Cyclin D1 immunoreactive band intensity (Figure 6.39).

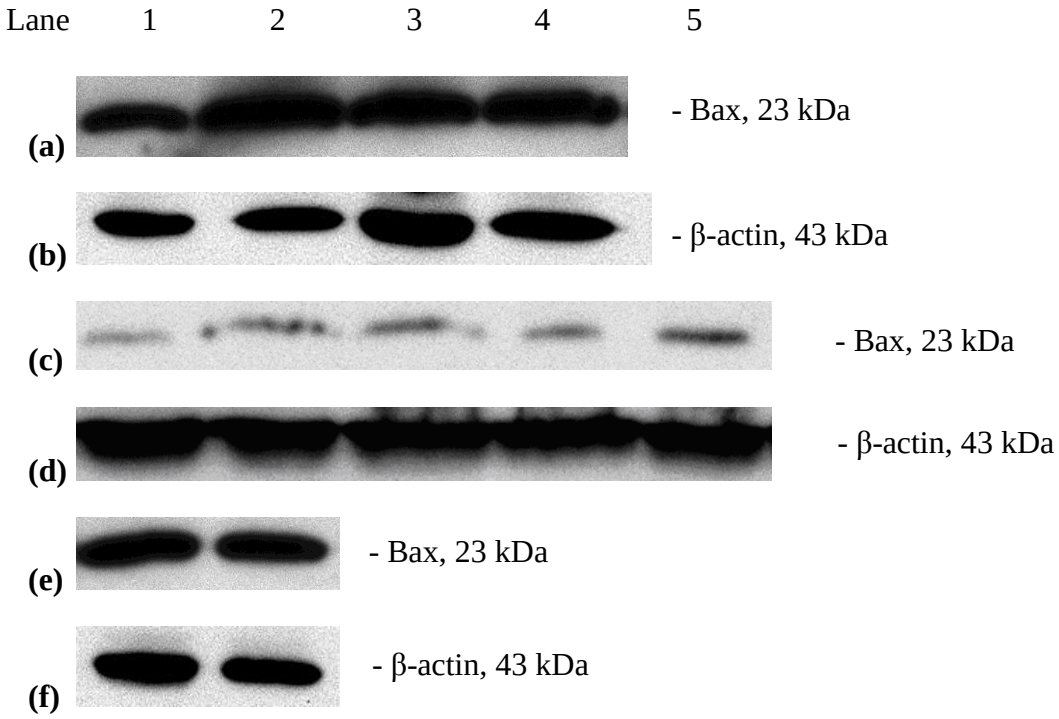


Figure 6.34. Western blotting analysis to detect pro-apoptotic Bax in phenolic acid treated and untreated HCT 116 cells. HCT 116 cell line was incubated with (a) caffeic acid, (c) ellagic acid, and (e) gallic acid at 0 to 800 µg/mL phenolic acid concentrations repectively. (b, d, and f) The level of β-actin in each sample was detected by a polyclonal anti-actin antibody (Sigma, UK). β-actin was used as internal standard to ensure the equal amounts of protein was loaded. Lane 1: 0 µg/mL, lane 2: 10 µg/mL, lane 3: 100 µg/mL, lane 4: 200 µg/mL, and lane 5: 400 µg/mL of caffeic, ellagic or gallic acid. kDa, molecular weight in thousands.

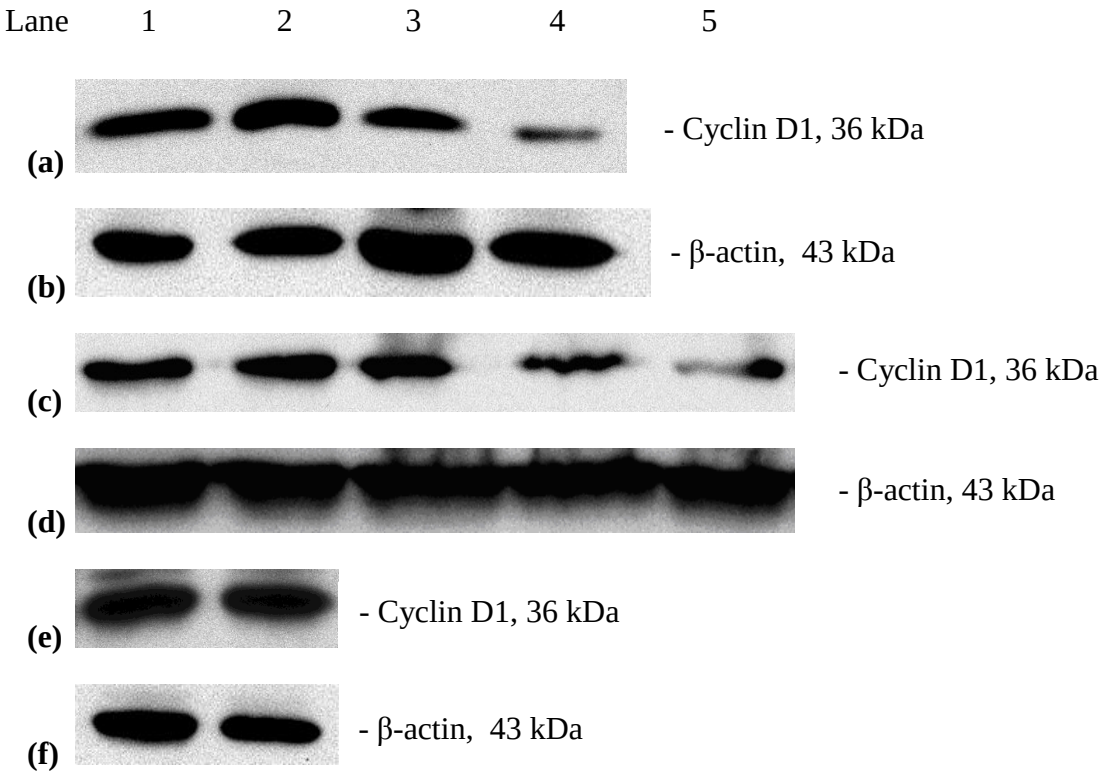


Figure 6.35. Western blotting analysis to detect Cyclin D1 in phenolic acid treated and untreated HCT 116 cells. HCT 116 cell line was incubated with (a) caffeic acid, (c) ellagic acid, and (e) gallic acid at 0 to 800 μg/mL phenolic acid concentrations. (b, d, and f) The level of β-actin in each sample was detected by a polyclonal anti-actin antibody (Sigma, UK). β-actin was used as internal standard to ensure the equal amounts of protein was loaded. Lane 1: 0 μg/mL, lane 2: 10 μg/mL, lane 3: 100 μg/mL, lane 4: 200 μg/mL, and lane 5: 400 μg/mL of caffeic, ellagic or gallic acid. kDa, molecular weight in thousands.

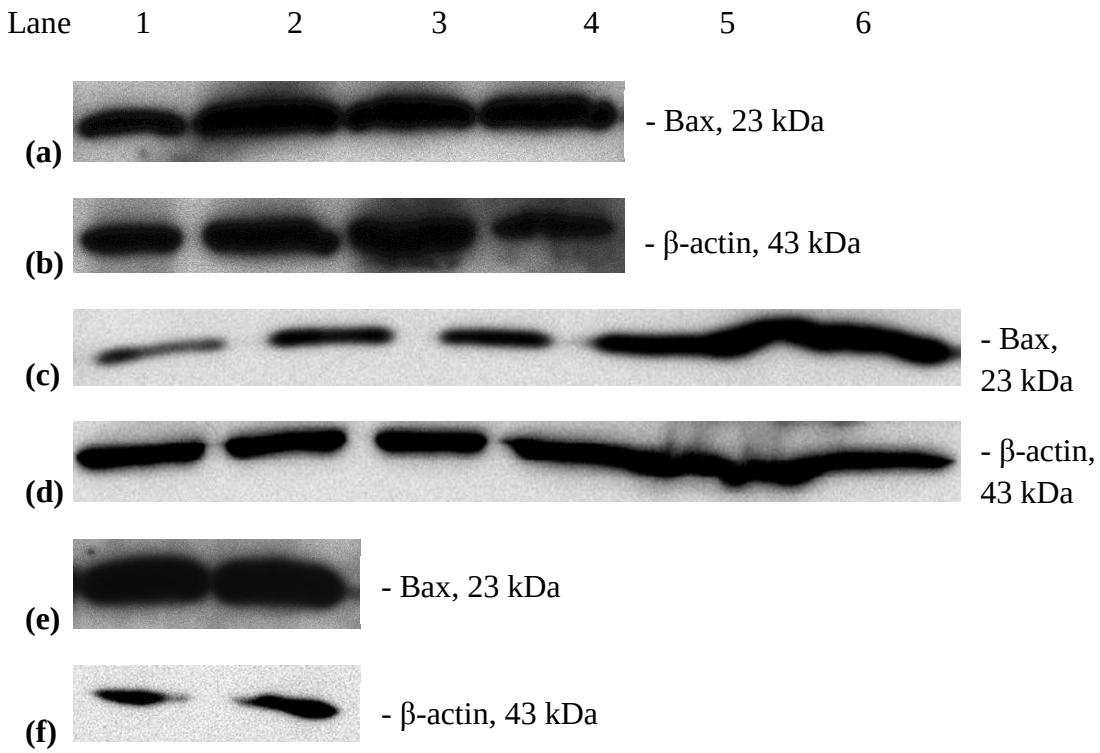


Figure 6.36. Western blotting analysis to detect pro-apoptotic Bax in phenolic acid treated and untreated HT-29 cells. HT-29 cell line was incubated with (a) caffeic acid, (c) chlorogenic acid, and (e) gallic acid at 0 to 800 μg/mL phenolic acid concentrations. (b, d, and, f) The level of β-actin in each sample was detected by a polyclonal anti-actin antibody (Sigma, UK). β-actin was used as internal standard to ensure the equal amounts of protein was loaded. Lane 1: 0 μg/mL, lane 2: 10 μg/mL, lane 3: 100 μg/mL, lane 4: 200 μg/mL, lane 5: 400 μg/mL, and lane 6: 800 μg/mL of caffeic, chlorogenic or gallic acid. kDa, molecular weight in thousands.

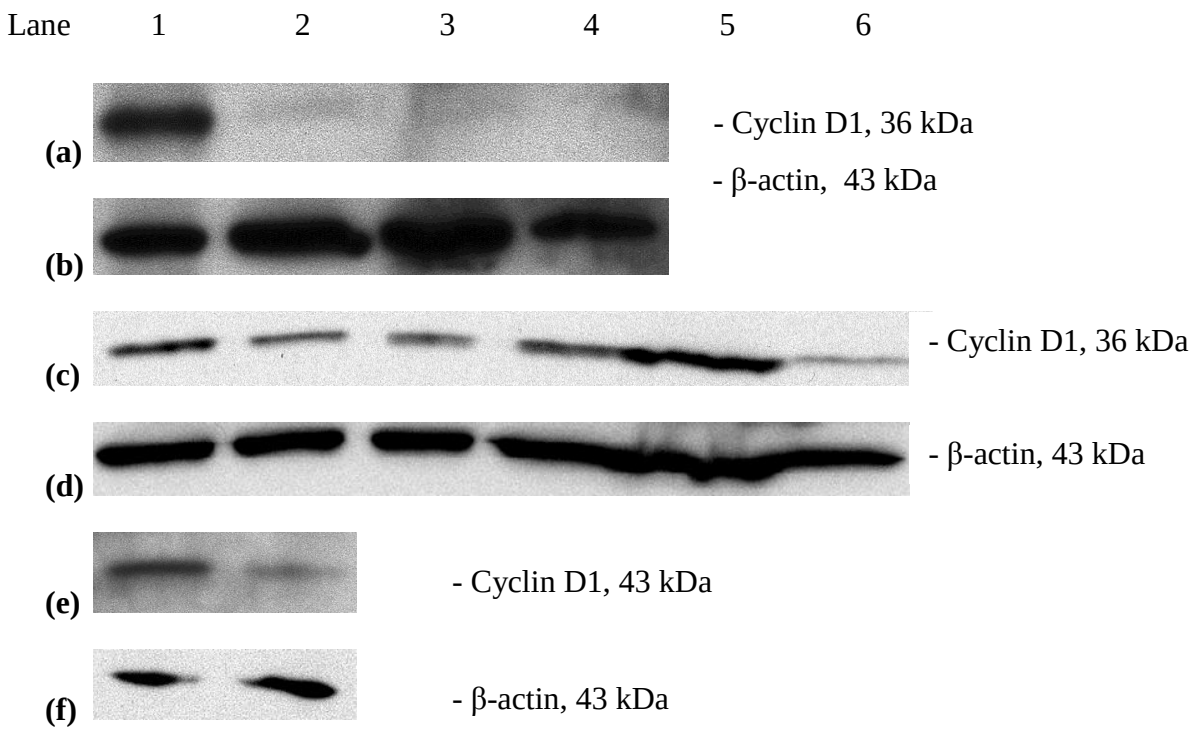


Figure 6.37. Western blotting analysis to detect Cyclin D1 in phenolic acid treated and untreated HT-29 cells. HT-29 cell line was incubated with (a) caffeic acid, (c) chlorogenic acid, and (e) gallic acid at 0 to 800 μg/mL phenolic acid concentrations. (b, d and, f) The level of β-actin in each sample was detected by a polyclonal anti-actin antibody (Sigma, UK). B-actin was used as internal standard to ensure the equal amounts of protein was loaded. Lane 1: 0 μg/mL, lane 2: 10 μg/mL, lane 3: 100 μg/mL, lane 4: 200 μg/mL, lane 5: 400 μg/mL, and lane 6: 800 μg/mL of caffeic, chlorogenic or gallic acid. kDa, molecular weight in thousands.

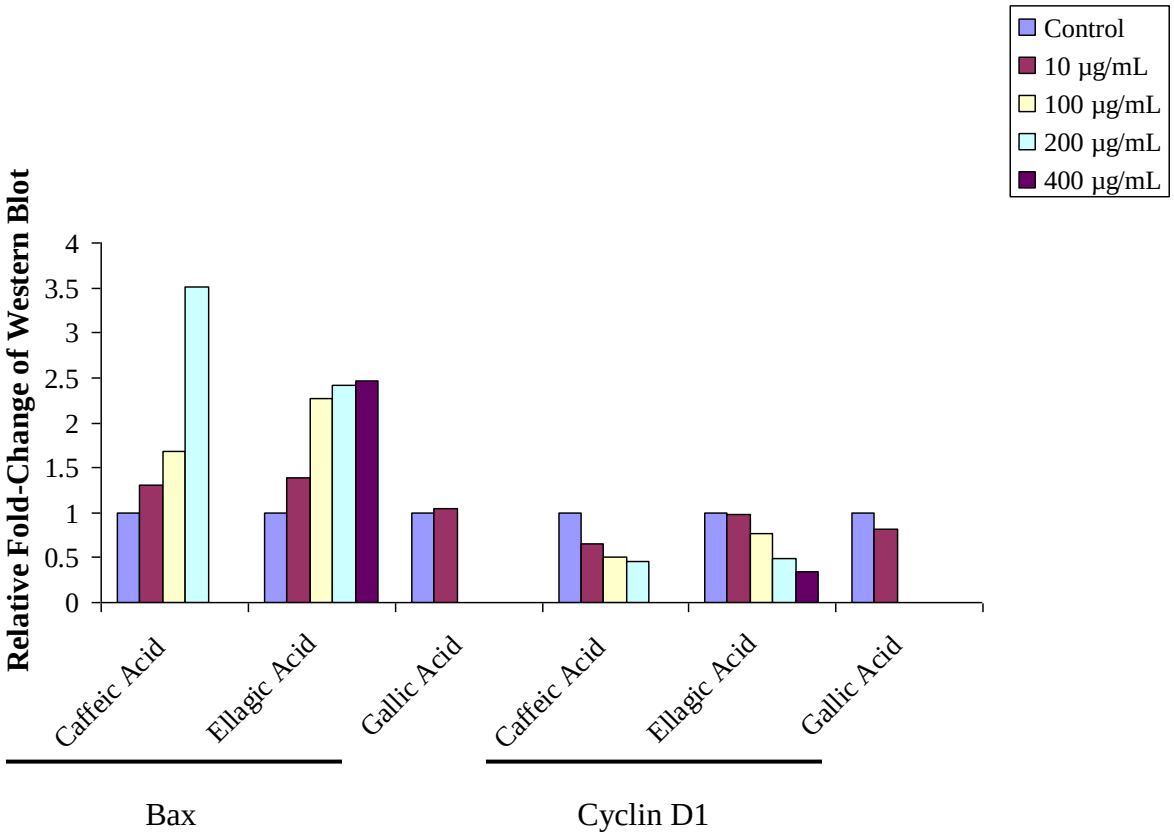


Figure 6.38. Relative quantitation of Western Blot of cell treatments from human colon cancer cell line HCT 116 probed with primary antibody against Bax or Cyclin D1.

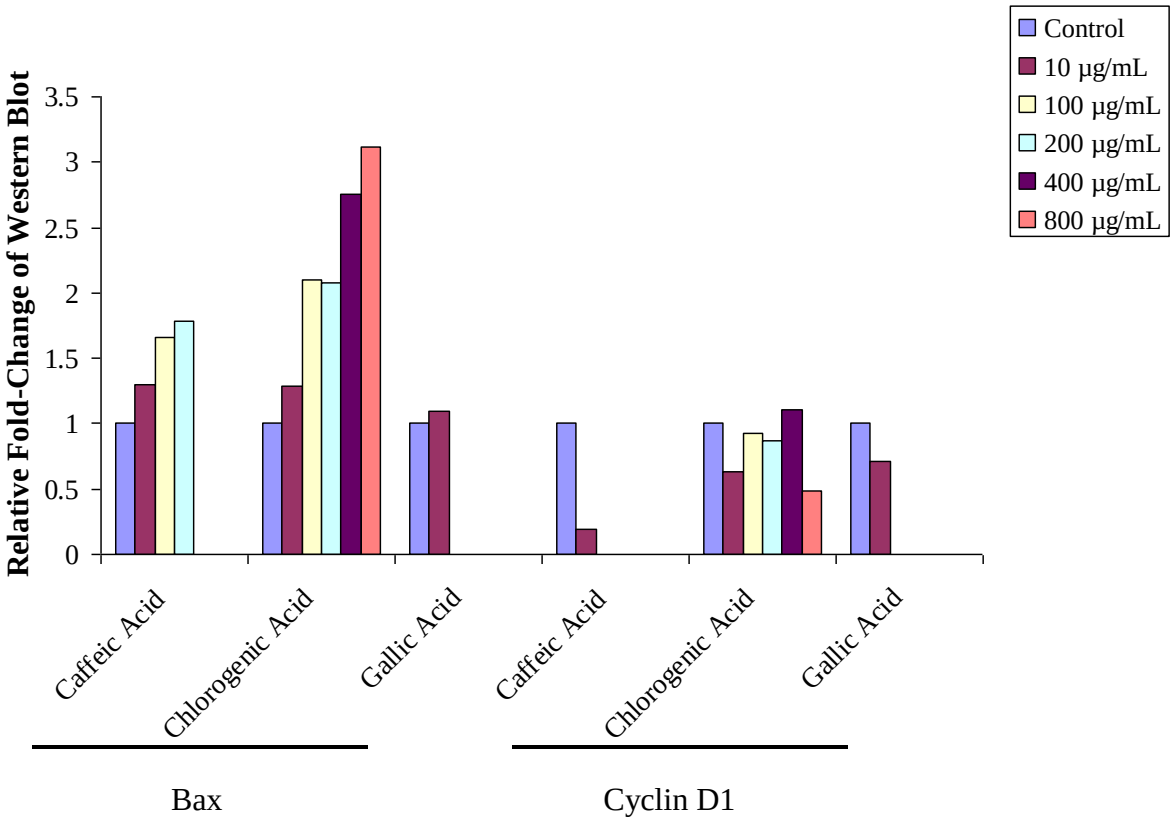


Figure 6.39. Relative quantitation of Western Blot of cell treatments from human colon cancer cell line HT-29 probed with primary antibody against Bax or Cyclin D1.

7.0 DISCUSSSION

Eleven phenolic acids used in this study have been shown to be present in wheat bran from six wheat cultivars belonging to six market classes (Verma et al 2009). Gallic and Ellagic acids show high anti-oxidant activities and chlorogenic acid is a metabolic product of caffeic acid, therefore, the three phenolic acids were also included in the study. The phenolic acids differed in their anti-oxidant potential as measured by the discoloration of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) (Beta *et al*., 2005) or 2, 2'-azino-bis(3-ethylbenzothiazone-6-sulphonic acid) (ABTS) discoloration (Trolox) equivalent anti-oxidant capacity (TEAC) assay (Re *et al*., 1999).

7.1 Two Different Human Colon Cells Were Used for the Study

One of the objectives of this study was to assess the utility of an *in vitro* cell culture-based screening assay to evaluate the efficacy of wheat bran phytochemicals in cancer prevention. The two human colon adenocarcinoma cell lines HT-29 and HCT 116 used in this study are different and have distinct advantages to indicate the molecular mechanism underlying colon cancer prevention by phenolic acids used in this study (Table 7.1).

7.2 Differential Growth Inhibitory Effects of Individual Phenolic Acids on HT-29 and HCT 116 Cell Lines

The individual phenolic acids differed in their ability to prevent the human colon cancer cell growth in HCT 116 and HT-29 as determined by calculating the half maximal inhibitory concentration (IC_{50}; mM) of phenolic acids at which only 50% of the cell survived. The effectiveness of individual phenolic acids to inhibit cell growth was divided into three categories: highly effective ($IC_{50} \leq 1$ mM), moderately effective (IC_{50} between 1 and 3 mM), and least effective ($IC_{50} \geq 3$ mM). For HT-29 cells the IC_{50} varied from as low as 0.06 mM for caffeic

acid to a high of 2.90 mM for salicylic acid (Figure 7.1). On the other hand for HCT-116 cell line, the IC_{50} values ranged from 0.15 mM for gallic acid to 5.79 mM for 4-hydroxybenzoic acid (Figure 7.2). Compared to benzoic acid derivatives, cinnamic acid derivatives had lower IC_{50} in both the cell lines, thus suggesting that these are more effective in inhibiting the growth of colon cancer cells. The growth of HCT 116 cells was inhibited the most by caffeic, ellagic, and gallic acids with IC_{50} of 0.22 mM, 0.17 mM, and 0.15 mM, respectively (Figures 7.2). On the other hand, to prevent the growth of HT-29 cells, caffeic, chlorogenic, and gallic acids are most effective, with IC_{50} at 0.06 mM, 0.28 mM, and 0.30 mM, respectively (Figure 7.1).

Table 7.1. Distinguishing characteristics of human colon cancer cell lines HT-29 and HCT 116 used in this study.

HT-29	**HCT 116**
Near triploid (modal chromosome number 68)	Diploid (modal chromosome number 46)
Frequent unbalanced chromosomal translocations and deletions	None observed
Display chromosomal instability pathway but stable at microsatellite level	Display chromosomal stability but show microsatellite instability
Normal β-catenin	Activating mutation in β-catenin gene
Exhibit two C-terminal-truncated APC protein of ~100 and 200 kDa	Wild type for APC
Normal ras pathway	K-ras mutation
Mutated non-functional p53	Normal p53

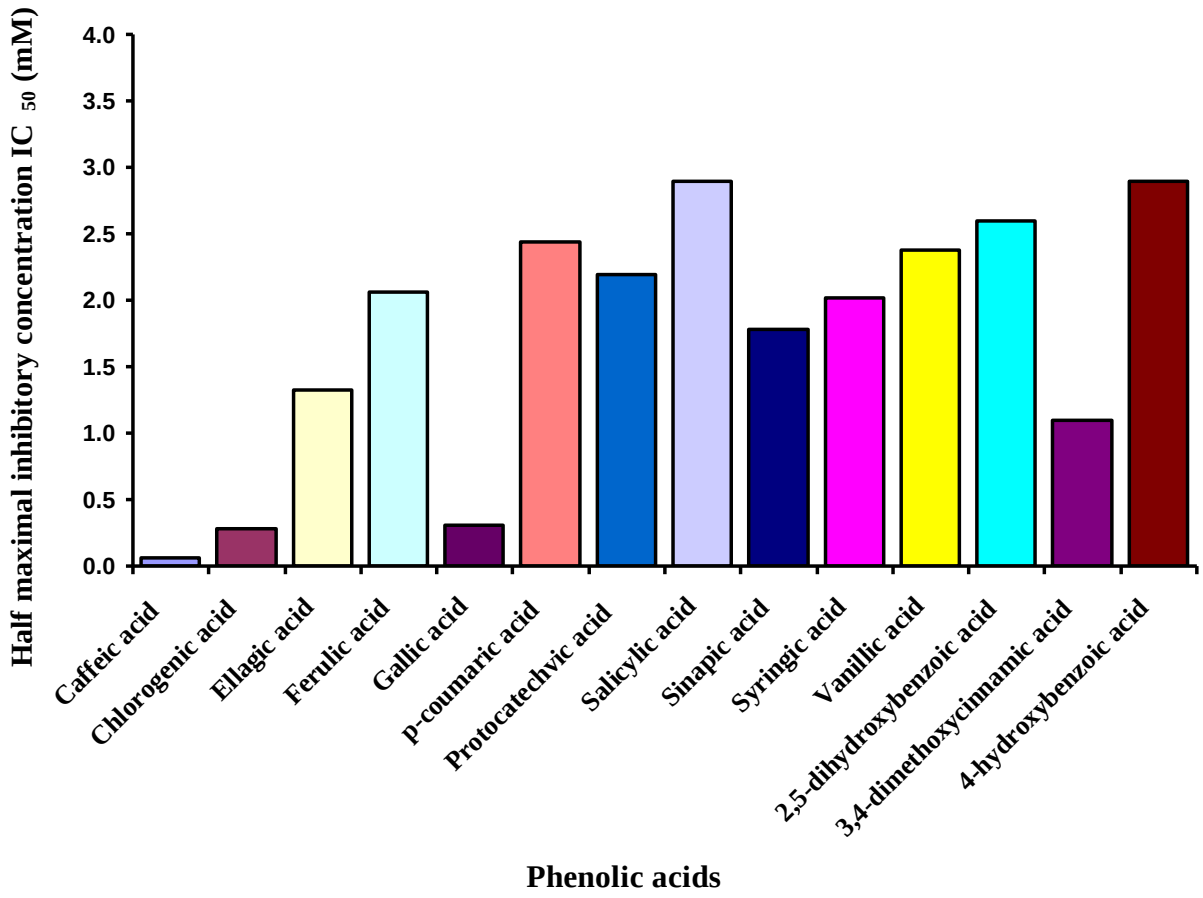


Figure 7.1. The half maximal inhibitory concentration (IC_{50}) of individual phenolic acids on HT-29 colon cancer cell line.

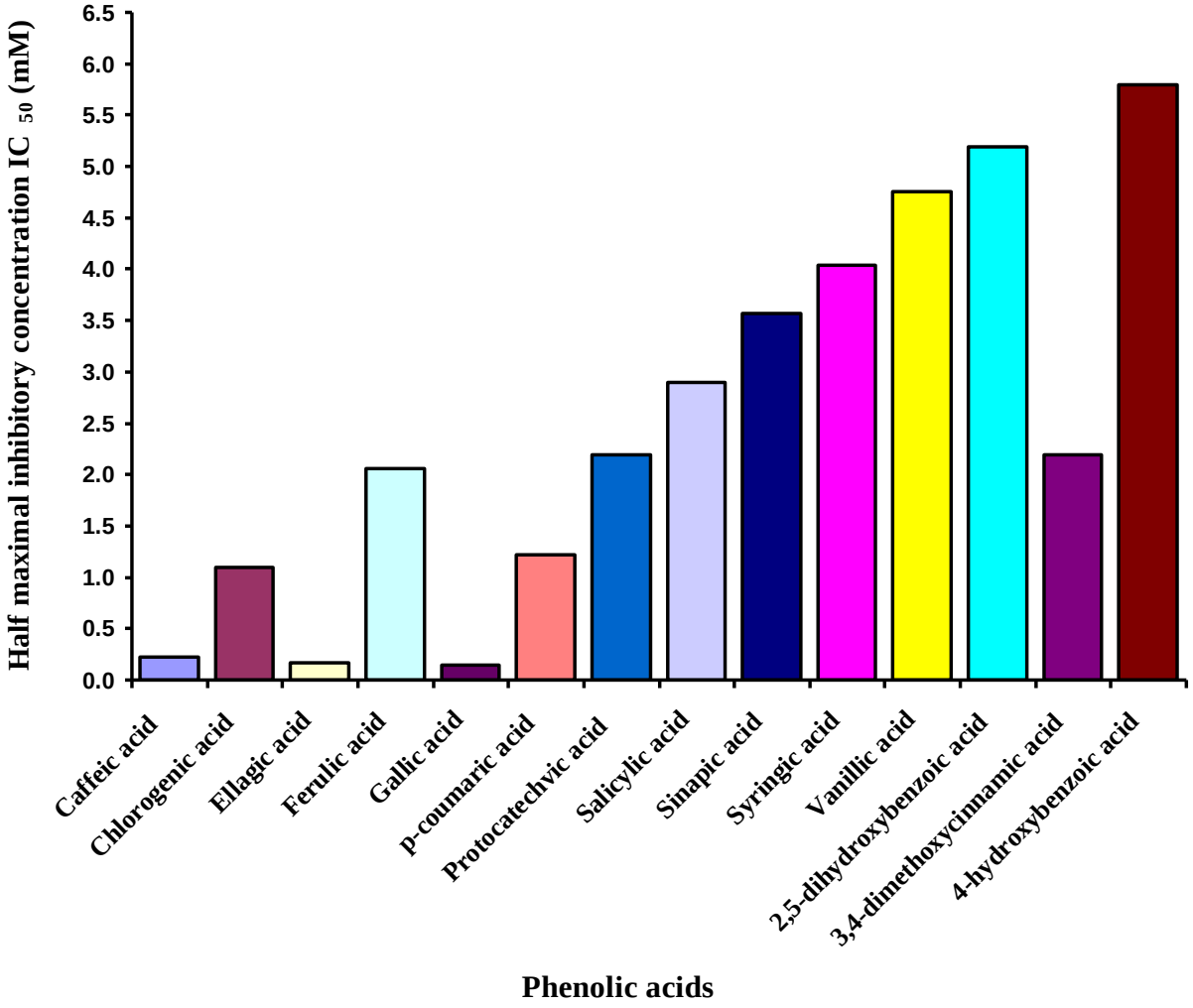


Figure 7.2. The half maximal inhibitory concentration (IC_{50}) of individual phenolic acids on HCT 116 colon cancer cell line.

7.3 Differential Effect of Phenolic Acids on Markers for Colon Cancer

A statistical analysis revealed that in both the cell lines HT-29 and HCT-116, phenolic acids differentially affected markers for cell proliferation, apoptosis, and cell inflammation. In general the phenolic acid treatment negatively correlated with cell proliferation and inflammatory markers, but positively correlated with apoptosis marker Bax (Table 7.2). Among the benzoic acid derivatives, ellagic acid, vanillic acid and 2, 5-dihydroxybenzoic acids showed highly significant ($p < 0.01$) negative correlations with the cell proliferation marker Ki67 (Table 7.2). In the cinnamic acid derivatives, caffeic acid, chlorogenic acid and ferulic acid showed highly significant negative correlation with Ki67 (Table 7.2). Ellagic acid, caffeic acid, chlorogenic acid and *p*-coumaric acid were significantly ($p < 0.01$) negatively correlated with inflammatory marker β-catenin (Table 7.2). However only caffeic acid and chlorogenic acid were highly significantly-correlated with another inflammatory marker iNOS (Table 7.2). COX-2 marker did not reveal statistically significant correlations with any of the phenolic acids tested. An apoptosis marker Bax was in general positively correlated with phenolic acid treatment of cells. Ellagic and ferulic acids were significantly ($p < 0.01$) positively correlated with Bax marker (Table 7.2). These results suggest that phenolic acid treatment may promote apoptosis and reduce cell proliferation and inflammation. Ellagic acid, gallic acid, caffeic acid, chlorogenic acid and ferulic acid showed at least one marker statistically significant in all the three processes studied (cell proliferation, apoptosis and inflammatory) and will be discussed in the following sections.

Table 7.2. Correlation coefficients to see the effects on both the cell lines together. (Pearson correlation were used to determine the effects; * represents $p < 0.05$; ** represents $p < 0.01$)

Phenolic acid	Cell number	Markers					
		Cell Proliferation		Apoptosis	Inflammatory		
		Cyclin D1	Ki67	Bax	β-catenin	COX-2	iNOS
Benzoic Acid Derivatives							
Ellagic Acid	-0.46	-0.39	-0.90**	0.72**	-0.74**	-0.02	-0.57
Gallic Acid	-0.62*	-0.45*	-0.58*	-0.59*	-0.61*	-0.39*	-0.62*
Protocatheuic Acid	-0.47	-0.10	-0.58*	0.20	-0.32	-0.03	-0.44
Salicylic Acid	-0.72**	-0.18	0.15	0.20	-0.61*	-0.02	-0.56
Vanillic Acid	-0.25	-0.17	-0.91**	-0.39	-0.42	-0.05	-0.63*
2, 5-Dihydroxybenzoic Acid	-0.25	-0.17	-0.91**	-0.39	-0.42	-0.05	-0.63*
4-Hydroxybenzoic Acid	-0.68*	-0.40	-0.25	0.55	-0.34	-0.05	-0.36
Cinnamic Acid Derivatives							
Caffeic Acid	-0.57	-0.57	-0.84**	-0.55	-0.87**	-0.43	-0.87**
Chlorogenic Acid	-0.69*	-0.51	-0.90**	-0.45	-0.88**	-0.39	-0.83**
p-Coumaric Acid	-0.48	-0.03	-0.53	0.45	-0.72**	-0.03	-0.43
Ferulic Acid	-0.61*	-0.27	-0.94**	0.71**	-0.65*	-0.03	-0.51
Sinapic Acid	-0.37	-0.49	-0.54	0.05	-0.26	-0.02	-0.69*
3, 4-Dimethoxycinnamic Acid	-0.71**	0.01	0.08	0.44	-0.55	-0.03	-0.62*

7.4 Ellagic Acid Affects Cell Proliferation, Apoptosis and Inflammatory Markers

Ellagic acid is a naturally occurring dietary phenolic acid which demonstrates a variety of anti-carcinogenic activities (Barch *et al*., 1996) and has been shown to reduce the risk of a number of carcinogen-induced tumors (Lesca, 1983; Mukhtar *et al*., 1984; Mandal and Stoner, 1990) through a number of different mechanisms (Mukhtar *et al*., 1984; Dixit *et al*., 1985; Dixit and Gold, 1986; Barch and Fox, 1988; Puchalski and Fahl, 1990; Barch and Rundhaugen, 1994; Barch *et al*., 1995). Ellagic acid is present in several berries, fruits and nuts including pomegranates and red berries such as raspberries, strawberries, and walnuts (Narayanan *et al*., 1999; Clifford and Scalbert, 2000; Fjaeraa and Nånberg, 2009). Ellagic acid is normally conjugated with a glycoside moiety such as glucose, arabinose, xilose and also forms part of polymeric molecules called ellagitannins (Clifford and Scalbert, 2000; Espín *et al*., 2007). In humans, ellagic acid is formed by hydrolysis of ellagitannins via lactonisation of hexahydroxydiphenic acid, and it is further metabolized by colonic microorganisms to yield urolithins which are more lipophilic (Clifford and Scalbert, 2000; Espín *et al*., 2007). Thus, ellagic acid is more readily absorbed over the intestinal mucosa into circulation.

Ellagic acid induces the expressions of phase II detoxification enzymes glutathione S-transferase (Barch *et al*., 1995) and NAD(P)H:quinine reductase (Barch and Rundhaugen, 1994). In addition, ellagic acid can also bind to DNA and inhibit the formation of O^6-methylguanine by methylating carcinogens (Dixit and Gold, 1986; Barch and Fox, 1988). This effect is consistent with the induction of O^6-methylguanine-DNA methyltransferase (MGMT) which rapidly removes AOM-induced promutagenic O^6-methylguanine DNA adducts and thus prevents oncogenic G to A point mutations (Zaidi *et al*., 1995; Pegg and Byers, 1992). There is growing

evidence that select mutagenic and lethal lesions involve the O^6 position of guanine (Sancar, 1995).

Functional groups of ellagic acid may play a role in its anti-carcinogenic activities (Barch *et al*., 1996). For example, the lactone groups are required to induce phase II enzymes and reduce carcinogen-induced formation of O^6-methylguanine, whereas both the 3- and 4-hydroxyl groups of ellagic acid are required to directly detoxify benzo[a]pyrene diolepoxide. Ellagic acid also shows anti-proliferative activity by inducing cell cycle arrest and/or apoptosis in several human cancer cell lines such as bladder T24, cervical carcinoma CaSki, leukemia MOLT-4, breast MCF-7, prostate DU 145, and colon cancer Caco-2 cells (Narayanan *et al*., 1999; Losso et al., 2004; Li *et al*., 2005; Mertens-Talcott *et al*., 2005; Larrosa *et al*., 2006). Ellagic acid showed differential anti-proliferative activity in Caco-2, MCF-7, and DU 145 cells, with Caco-2 cells were the most sensitive cells followed by DU 145 and MCF-7 cells (Losso *et al*., 2004). The anti-carcinogenic effect of dietary ellagitannins could be mainly due to their hydrolysis product, ellagic acid, which induced cell-cycle arrest as well as apoptosis via mitochondrial pathway in Caco-2 colon cancer cells (Larrosa *et al*., 2006). Ellagic acid treatment of Caco-2 cells downregulated cyclin A and B1 and upregulated cyclin E and arrested cell cycle in the S phase.

In this study, ellagic acid showed the strongest inhibitory effect on HCT 116 cells, with IC_{50} = 0.17 mM. In both the cell lines HT-29 and HCT 116, ellagic acid upregulated Bax and downregulated Cyclin D1 and β-catenin and iNOS proteins in a concentration dependent manner. This suggests that ellagic acid reduced cell proliferation and inflammatory pathways but promotes apoptosis. These results concur with those obtained with crude pomegranate fruit extract, containing ellagic acid among others, inhibited cell growth and induced apoptosis in a dose dependent manner in androgen-insensitive prostate cancer PC3 cells (Malik et al 2005).

The two apoptosis markers Bax and Bak showed increased expression, while the cell proliferation markers cyclins D1, D2, and E and cdk 2, 4, and 6 expression was decreased. In another animal study, ellagic acid inhibited DMH-induced activation of PI3K/Akt and influenced the downstream Bcl-2 family proteins (Umesalma and Sudhandiran 2011). Administration of ellagic acid in rats upregulated Bax and caspases-3 which elevated cytochrome c levels and finally led to cell death.

7.5 Effect of Gallic Acid on HT-29 and HCT 116 Cells

Gallic acid (3, 4, 5-trihydroyl-benzoic acid) is present in several plants, fruits and foods such as cereals, legumes, tea leaves, and whole grain bread (Salucci *et al*., 2002, You and Park, 2010, Djordjevic *et al*., 2011). Gallic acid is readily absorbed by humans, as consumption of gallic acid rich food and gallic acid tablet, caused several micromoles of free gallic acid and glucuronidated forms of gallic acid and its main metabolite, 4-O-methyl gallic acid, in human blood plasma (Shahrzad *et al*., 2001; You and Park, 2010).

Gallic acid induces programmed cell death in various tumor cell lines like in leukemia, lung, breast, colon and gastric cancer (Inoue *et al*., 2000; Ohno *et al*., 1999; Faried *et al*., 2007). Gallic acid inhibits the growth of colon cancer cell lines (Caco-2, HT-29, Colo-201), and *in vivo* studies on colon carcinogenesis (Salucci *et al*., 2002; Faried *et al*., 2007). In Wistar rats administration of gallic acid restored colon specific carcinogen induced histological changes and xenobiotic-metabolizing enzyme systems, thus reducing the incidence of colon cancer (Giftson *et al*., 2011).

Our data showed that gallic acid-treated cells reduced markers for all three processes — cell proliferation, apoptosis and inflammatory pathway — which is in agreement with previous studies that gallic acid is associated with induced anti-proliferation and/or apoptotic activities in

various cancers (Inoue *et al*., 2000; Salucci *et al*., 2002; Faried *et al*., 2007; You and Park, 2010). Gallic acid has very low $IC_{50,}$ thus confirming its high potency and suggests that gallic acid could be a very effective chemopreventive.

7.6 Effect of Caffeic Acid on HT-29 and HCT 116 Cells

Caffeic acid is one of the major representative hydroxycinnamic acids, and occurs in foods mainly as an ester with quinic acid (Boerjan *et al*., 2003). The hydroxylation produces caffeic acid ester of shikimic acid, which converts to chlorogenic acid (Boerjan *et al*., 2003). Coffee contains high level of caffeic acid. The caffeic acid content of a 200 mL cup of coffee has been reported to range about 35 to 175 mg (Clifford, 2000).

Caffeic acid has been shown to inhibit growth of several cancers such as hepatocarcinoma HepG2 (Chung *et al*., 2004) and human ovarian cancer A2780 cells (Tai *et al*., 2010). 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced epidermal tumors in mice were reduced by 28% and 35% when simultaneously administered with chlorogenic acid or caffeic acid, respectively (Huang *et al*., 1988).

To our knowledge, studies showing the anti-proliferation and pro-apoptotic activity of caffeic acid on both HT-29 and HCT 116 human colon cancer cell lines are scarce and not well-documented. Caffeic acid had growth inhibitory effect on both human colon cancer HT-29 and HCT 116 cells. However, caffeic acid showed the most anti-proliferation effect on HT-29 cells compared to HCT 116 cells, anti-proliferative IC_{50} of 0.06 mM and 0.22 mM respectively. In our study, caffeic acid induced anti-proliferation and apoptotic activities in both cell lines are associated with downregulation of Cyclin D1 and upregulation of Bax.

7.7 Effect of Chlorogenic Acid on HT-29 Cells

Chlorogenic acids are a family of esters formed between a *trans*-cinnamic acid such as caffeic acid or ferulic acid and quinic acid which has axial hydroxyls on carbon 1 and 3 and equatorial hydroxyls on carbon 4 and 5 (Clifford, 2000). Intestinal microorganisms hydrolyze chlorogenic acid into various aromatic acid metabolites including caffeic acid and quinic acid (Gonthier *et al*., 2003). It is widespread in plants, fruits, grains, and vegetables (Clifford, 2000; Verma *et al*., 2009). For example, coffee is one of the beverages containing large amount of phenolic acids, especially chlorogenic acid, which in a single cup (200 mL) of coffee varies between 70 and 350 mg (Clifford, 2000).

Chlorogenic acid shows anti-proliferative activity and induces apoptosis in several human cancer cell lines such as oral squamous cell carcinoma HSC-2, salivary gland tumor HSG (Jiang *et al*., 2000), and human ovarian cancer A2780 cells (Tai *et al*., 2010). Chlorogenic acid exhibits anti-mutagenic, anti-carcinogenic, anti-oxidant activities, and ability to scavenge reactive oxygen species (Rice-Evans *et al*., 1996).

To our knowledge, there are few if any reports showing the anti-proliferation and pro-apoptotic activity of chlorogenic acid on both HT-29 and HCT 116 human colon cancer cell lines. Chlorogenic acid was more effective in preventing HT-29 cells than that of the HCT 116 cells. The anti-proliferation IC_{50} concentrations for chlorogenic acid on HT-29 and HCT 116 cells were 0.28 mM and 1.10 mM respectively. In our study, the chlorogenic acid upregulated Bax and downregulated Cyclin D1 proteins, suggesting it induced apoptosis and inhibited cell proliferative activity.

7.8 Anti-oxidant Capacities of Phenolic Acids

The eleven phenolic acids' concentrations, profiles, and anti-oxidant capacities (Table 7.5) from the bran of six wheat cultivars were determined (Verma *et al*., 2009) and were used in this study to screen their effects on prevention colon cancer cell growth. The two anti-oxidant assays also show differential activity, suggesting that phenolics react differentially to the DPPH and ABTS radicals (Verma *et al*., 2009).

The benzoic acid derivatives ellagic acid and gallic acid had very high anti-oxidant activity as measured by DPPH assay and also had very low IC_{50} values suggesting these are potent agents to reduce the growth of colon cancer cells (Table 7.3). However, 2, 5-dihydroxy benzoic acid which showed the highest DPPH based anti-oxidant activity showed very high IC_{50} for both the cell lines. In the cinnamic acid derivatives both caffeic acid and chlorogenic acid show high DPPH based anti-oxidant activity and low IC_{50} in both cell lines, but more in HT-29 as compared to HCT 116 (Table 7.3). These results showed some relationship between anti-oxidant activity and the inhibitory effect on the growth of the two cell lines. However, this limited study provided no definite conclusions regarding the structure of phenols and anti-oxidant activity and inhibition of cell growth.

7.9 General Conclusions

A major finding of this study is that both the cell lines HT-29 and HCT 116 can be used to screen the anti-carcinogenic activity of plant-based chemical compounds. The two cell lines differed in their genetics and some biochemical markers associated with cancer development. However, detailed marker analysis clearly showed that pro-apoptosis marker was increased in both the cell lines in response to most of the phenolic acids studied. Bax marker expression also increased in HT-29 colon cancer lines treated with bilberry (*Vaccinium myrtillus*) or cloudberry

(*Rubus chamaemorous*) extracts (Wu *et al*., 2007). These results suggest that phenolic acid treatment predisposes the cells to apoptosis as one of the mechanisms to prevent colon cancer.

Table 7.3. Anti-oxidant activity of 50 µg ml^{-1} phenolic acid standards. Anti-oxidant is expressed as percent discolouration of 2, 2-diphenyl-1-picrylhydrazyl (DPPH). Negative number indicates increased colouration of the radical solution. (*Data adapted from Verma *et al*., 2009)

Phenolic acids	Molecular Weight (g/mol)	IC_{50} (mM)		Anti-oxidant Value *	
		HT-29	HCT 116	DPPH	ABTS
Benzoic Acid Derivatives					
Ellagic acid	302.19	1.32	0.17	49.0	0.12
Gallic acid	170.12	0.30	0.15	60.2	1.17
Protocatechuic acid	182.17	2.20	2.20	17.2	0.48
Salicylic acid	138.12	2.90	2.90	-6.4	0.003
Syringic acid	198.17	2.02	4.04	7.3	0.45
Vanillic acid	168.15	2.38	4.76	-5.8	0.35
2, 5-Dihydroxybenzoic acid	154.12	2.60	5.19	78.9	0.48
4-Hydroxybenzoic acid	138.12	2.90	5.79	4.1	-0.019
Cinnamic Acid Derivatives					
Caffeic acid	180.16	0.06	0.22	26.9	0.39
Chlorogenic acid	363.32	0.28	1.10	22.0	0.17
p-Coumaric acid	164.16	2.44	1.22	1.5	0.47
Ferulic acid	194.18	2.06	2.06	8.1	0.64
Sinapic acid	224.21	1.78	3.57	17.4	0.41
3, 4-Dimethoxycinnamic acid	182.17	1.10	2.20	1.2	-0.02

The cell proliferation markers were negatively correlated to phenolic acid treatment, suggesting that phenolic acid treatment inhibited cell division. Cell inflammatory markers were mostly negatively correlated with phenolic acid treatment; however, the trends were not consistent, thus indicating that phenolic acid treatment affected different cell inflammatory pathways. Higher anti-oxidant activity to some extent influenced cell growth of both the cell lines. However, the results were not conclusive to draw some definitive conclusions.

7.10 Future Directions

These studies have provided some very interesting observations and established a protocol to study the effect of phenolic acids or other plant constituents on two different colon cancer lines. Definite conclusions on the molecular mechanisms underlying cell growth inhibition by the phenolic acids could not be elucidated from this study. Immunohistochemical studies are good indicators of the presence of marker proteins, but to identify the molecular mechanism underlying the response, precise gene expression studies using RNA gel blot (Northern hybridization) and or real-time quantitative PCR will be needed to be done. To understand the role of wheat bran products in colon cancer prevention, studies should be carried out with pre-analyzed wheat bran extracts using the two colon cancer lines and methods developed in this project. Different wheat varieties have different bran compositions, with different ant-oxidant activities. Another important study that can be done is to investigate the interaction of one or more phenolic acids in prevention of growtn of colon cancer cell lines.

8.0 REFERENCES

AACC Dietary Fiber Technical Committee. (2001). Definition of dietary fiber. Cereal Food World *46*, 112.

Aaltonen, L.A., Salovaara, R., Kristo, P., Canzian, F., Hemminki, A., Peltomäki, P., Chadwick, R.B., Kääriänen, H., Eskelinen, M., Järvinen, H., Mecklin, J.-P., de la Chapelle, A., Percesepe, A., Ahtola, H., Härkönen, N., Julkunen, R., Kangas, E., Ojala, S., Tulikoura, J., and Valkamo, E. (1998). Incidence of hereditary nonpolyposis colorectal cancer and the feasibility of molecular screening for the disease. N. Engl. J. Med. *338*, 1481-1487.

Abdel-Aal, E.S.M., Hucl, P., Sosulski, F.W., Graf, R., Gillot, C., and Pietrzak, L. (2001). Screening spring wheat for midge resistance in relation to ferulic acid content. J. Agri. Food Chem. *49*, 3559-3566.

Abdel-Rahman, W.M., Mecklin, J-P., and Peltomäki, P. (2006). The genetics of HNPCC: application to diagnosis and screening. Crit. Rev. Oncol. Hematol. *58*, 208-220.

Adrain, C., and Martin, S.J. (2001). The mitochondrial apoptosome: a killer unleashed by the cytochrome seas. Treads Biochem. Sci. *26*, 390-397.

Ahn, B., and Oshima, H. (2001). Suppression of intestinal polyposis in $APC^{(Min/+)}$ mice by inhibiting nitric oxide production. Cancer Res. *61*, 8357-8360.

Alabaster, O., Tang, Z., and Shivapurkar, N. (1997). Inhibition by wheat bran cereals of the development of aberrant crypt foci and colon tumors. Food Chem. Toxicol. *35*, 517-522.

Ambs, S., Merriam, W.G., Bennett, W.P., Felley-Bosco, E., Ogunfusika, M.O., Oser, S.M., Klein, S., Shields, P.G., Billiar, T.R., and Harris, C.C. (1998). Frequent nitric oxide synthase-2 expression in human colon adenomas: implication for tumor angiogenesis and colon cancer progression. Cancer Res. *58*, 334-341.

Anderson, J.W., Baird, P., Davis, R.H. Jr., Ferreri, S., Knudtson, M., Koraym, A., Waters, V., and Williams, C.L. (2009). Health benefits of dietary fibers. Nutr. Rev. *67*, 188-205.

Arakawa, H. (2004). Netrin-1 and its receptors in tumorigenesis. Nat. Rev. Cancer *4*, 978-987.

Araki, Y., Okamura, S., Hussain, S.P., Nagashima, M., He, P., Shiseki, M., Miura, K., and Harris, C.C. (2003). Regulation of cyclooxygenase-2 by the Wnt and Ras pathways. Cancer Res. *63*, 728-734.

Arber, N., Doki, Y., Han, E.K., Sgambato, A., Zhou, P., Kim, N.H., Delohery, T., Klein, M.G., Holt, P.R., and Weinstein, I.B. (1997). Antisense to cyclin D1 inhibits the growth and tumorigenicity of human colon cancer cells. Cancer Res. *57*, 1569-1574.

Arber, N., Hibshoosh, H., Moss, S.F., Sutter, T., Begg, M., Wang, S., Weinstein, I.B., and Holt, P.R. (1996). Increased expression of cyclin D1 is an early event in multistage colorectal carcinogenesis. Gastroenterology *110*, 669-674.

Archer, S.Y., Meng, S., Shei, A., and Hodin, R.A. (1998). P21 (WAF1) is required for butyrate-mediated growth inhibition of human colon cancer cells. Proc. Natl. Acad. Sci. USA *95*, 6791-6796.

Arnold, C.N., Goel, A., Niedzwiecki, D., Dowell, J.M., Wasserman, L., Compton, C., Mayer, R.J., Bertagnolli, M.M., and Boland, C.R. (2004). APC promoter hypermethylation contributes to the loss of APC expression in colorectal cancers with allelic loss in 5q. Cancer Biol. Ther. *3*, 960-964.

Asp. N.-G., Johansson, C.G., Hallmer, H., and Siljestrom. M. (1983). Rapid enzymatic assay of insoluble and soluble dietary fiber. J. Agric. Food Chem. *31*, 476-482.

Baker, S.M., Bronner, C.E., Zhang, L., Plug, A.W., Robatzek, M., Warren, G., Elliot, E.A., Yu, J., Ashley, T., Arnheim, N., Flavell, R.A., and Liskay, R.M. (1995). Male mice defective in the DNA mismatch repair gene PMS2 exhibit abnormal chromosome synapsis in meiosis. Cell *82*, 309-319.

Baker, S.M., Plug, A.W., Prolla, T.A., Bronner, C.E., Harris, A.C., Yao, X., Christie, D.-M., Monell, C., Arnheim, N., Bradley, A., Ashley, T., and Liskay, M. (1996). Involvement of mouse Mlh1 in DNA mismatch repair and meiotic crossing over. Cell *13*, 336-342.

Bach, S.P., Renehan, A.G., and Potten, C.S. (2000). Stem cells: the intestinal stem cell as a paradigm. Carcinogenesis *21*, 469-476.

Backus, H.H., Van Groeningen, C.J., Vos, W., Dukers, D.F., Bloemena, E., Wouters, D., Pinedo, H.M., and Peters, G.J. (2002). Differential expression of cell cycle and apoptosis related proteins in colorectal mucosal, primary colon tumors and liver metastases. J. Clin. Pathol. *55*, 206-211.

Baldassarre, G., Nicoloso, M.S., Schiappacassi, M., Chimienti, C., and Belletti, B. (2004). Linking inflammation to cell cycle progression. Curr. Pharm. Des. *10*, 1653-1655.

Barch, D.H., and Fox, C.C. (1988). Selective inhibition of methylbenzylnitrosamine induced formation of esophageal O^6-methylguanine by dietary ellagic acid in rats. Cancer Res. *48*, 7088-7092.

Barch, D.H., and Rundhaugen, L.M. (1994). Ellagic acid induces NAD(P)H:quinine reductase through activation of the antioxidant regulatory element of the rat NAD(P)H:quinine reductase gene. Carcinogenesis *15*, 2065-2068.

Barch, D.H., Rundhaugen, L.M., and Pillay, N.S. (1995). Ellagic acid induces transcription of the rat gluthathione S-transferase Ya gene. Carcinogenesis *16*, 665-668.

Barch, D.H., Rundhaugen, L.M., Stoner, G.D., Pillay, N.S., and Rosche, W.A. (1996). Structure-function relationships of the dietary anticarcinogen ellagic acid. Carcinogenesis *17*, 265-269.

Bartkova, J., Lukas, J., Strauss, M., and Bartek, J. (1994). The PRAD-1/cyclin D1 oncogene product accumulates aberrantly in a subset of colorectal carcinomas. Int. J. Cancer *58*, 568-573.

Baublis, A.J., Clydesdale, F.M., and Decker, E.A. (2000). Antioxidants in wheat-based breakfast cereals. Cereal Food World 45, 71-74.

Baublis, A.J., Lu, C., Clydesdale, F.M., and Decker, E.A. (2002). Potential of wheat-based breakfast cereals as a source of dietary antioxidants. J. Am. Coll. Nutr. *19*, 308S-311S.

Bedi, A., Pasricha, P.J., Akhtar, A.J., Barber, J.P., Bedi, G.C., Giardiello, F.M., Zehnbauer, B.S., Hamilton, S.R., and Jones, R.J. (1995). Inhibition of apoptosis during development of colorectal cancer. Cancer Res. *55*, 1811-1816.

Behrens, J. (2005). The role of the Wnt signalling pathway in colorectal tumorigenesis. Biochem. Soc. Trans. *33*, 672-675.

Bergman, E.N. (1990). Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. Physiol. Rev. *70*, 567-590.

Beroud, C., and Soussi, T. (1996). APC gene: database of germline and somatic mutations in human tumors and cell line. Nucleic Acids Res. *24*, 121-124.

Beta, T., Nam, S., Dexter, J.E., and Sapirstein, H.D. (2005). Phenolic content and antioxidant activity of pearled wheat and roller-milled fractions. Cereal Chem. *82*, 390-393.

Bienz, M., and Clevers, H. (2000). Linking colorectal cancer to Wnt signaling. Cell *103*, 311-320.

Bingham, S.A., Day, N.E., Luben, R., Ferrari, P., Slimani, N., Norat, T., Clavel-Chapelon, F., Kesse, E., Nieters, A., Boeing, H., Tjønneland, A., Overvad, K., Martinez, C., Dorronsoro, M., Gonzalez, C.A., Key, T.J., Trichopoulou, A., Naska, A., Vineis, P., Tumino, R., Krogh, V., Bueno-de-Mesquita, H.B., Peeters, P.H.M., Berglund, G., Hallmans, G., Lund, E., Skeie, G., Kaaks, R., and Riboli, E. (2003). Dietary fibre in food and protection against colorectal cancer in the European Prospective Investigation into Cancer and Nutrition (EPIC): an observational study. Lancet *361*, 1496-1501.

Boerjan, W., Ralph, J., and Baucher, M. (2003). Lignin biosynthesis. Annu. Rev. Plant Biol. *54*, 519-546.

Botteri, E., Iodice, S., Bagnardi, V., Raimondi, S., Lowenfels, A.B., and Maisonneuve, P. (2008). Smoking and colorectal cancer: a meta-analysis. JAMA *300*, 2765-2778.

Bredesen, D.E., Mehlen, P., and Rabizadeh, S. (2005). Receptors that mediate cellular dependence. Cell Death Differ. *12*, 1031-1043.

Bronner, M.P., Culin, C., Reed, J.C., and Furth, E.E. (1995). The bcl-s proto-oncogene and the gastrointestinal tumor progression model. Am. J. Pathol. *146*, 20-26.

Bruce, W.R. (1987). Recent hypotheses for the origin of colon cancer. Cancer Res. *47*, 4237-4242.

Budd, R. (2001). Activation-induced cell death. Curr. Opin. Immunol. *13*, 356-362.

Burkitt, D.P. (1970). Relationship as a clue to causation. Lancet *2*, 1237-1240.

Burkitt, D.P. (1971). Epidemiology of cancer of the colon and rectum. Cancer *28*, 3-13.

Camps, J., Morales, C., Prat, E., Ribas, M., Capellà, G., Egozcue, J., Peinado, M.A., and Miró, R. (2004). Genetic evolution in colon cancer KM12 cells and metastatic derivatives. Int. J. Cancer *110*, 869-874.

Castellone, M.D., Teramoto, H., and Gutkind, J.S. (2006). Cyclooxygenease-2 and colorectal cancer chemoprevention: the β-catenin connection. Cancer Res. *66*, 11085-11088.

Center, M.M., Jemal, A., Smith, R.A., and Ward, E. (2009a). Worldwide variations in colorectal cancer. CA Cancer J. Clin. *59*, 366-378.

Center, M.M., Jemal, A., and Ward, E. (2009b). International trends in colorectal cancer incidence rates. Cancer Epidemiol. Biomark. Prev. *18*, 1688-1694.

Chan, A.T., Ogino, S., and Fuchs, C.S. (2007). Aspirin and thr risk of colorectal cancer in relation to the expression of COX-2. N. Engl. J. Med. *356*, 2131-2142.

Chang, W.C.L., Chapkin, R.S., and Lupton, J.R. (1997). Predictive value of proliferation, differentiation and apoptosis as intermediate markers for colon tumorigenesis. Carcinogenesis *18*, 721-730.

Chung, T.-W., Moon, S.-K., Chang, Y.-C., Ko, J.-H., Lee, Y.-C., Cho, G., Kim, S.-H., Kim, J.-G., and Kim, C.-H. (2004). Novel and therapeutic effect of caffeic acid and caffeic acid phenyl ester on hepatocarcinoma cells: complete regression of hepatoma growth and metastasis by dual mechanism. Faseb J. *18*, 1670-1681.

Cianchi, F., Cortesini, C., Fantappiè, O., Messerini, L., Schiavone, N., Vannacci, A., Nistri, S., Sardi, I., Baroni, G., Marzocca, C., Perna, F., Mazzanti, R., Bechi, P., and Masini,

E. (2003). Inducible nitric oxide synthase expression in human colorectal cancer: correlation with tumor angiogenesis. Am. J. Pathol. *162*, 793-801.

Clarke, A.R., Gledhill, S., Hooper, M.L., Bird, C.C., and Wyllie, A.H. (1994). P53 dependence of early apoptotic and proliferative responses within the mouse intestinal epithelium following gamma-irradiation. Oncogene *9*, 1767-1773.

Clevers, H. (2006). Colon cancer: understating how NSAIDs work. N. Engl. J. Med. *354*, 761-763.

Clifford, M.N. (2000). Chlorogenic acids and other cinnamates – nature, occurrence and dietary burden, absorption and metabolism. J. Sci. Food Agric. *80*, 1033-1043.

Clifford, M.N., and Scalber, A. (2000). Ellagitannins – nature, occurrence and dietary burden. J. Sci. Food Agric. *80*, 118-125.

CNFSDU-WHO/FAO. Cordex Alimentarius Commission (2008). 30th Session. Available from http://www.codexalimentarius.net/web/archives.jsp?yearZ09. ALINORM 09/32/26. Para 27-54 and page 46.

Cook, S.I., and Sellin, J.H. (1998). Review article: short chain fatty acids in health and disease. Aliment Pharmacol Ther. *12*, 499-507.

Corpet, D.E., and Pierre, F. (2005). How good are rodent models of carcinogenesis in predicting efficacy in humans? A systematic review and meta-analysis of colon chemoprevention in rats, mice and men. Eur. J. Cancer *41*, 1911-1922.

Costabile, A., Klinder, A., Fava, F., Napolitano, A., Foglinao, V., Leonard, C., Gibson, G.R., and Tuohy, K.M. (2008). Whole-grain wheat breakfast cereal has a prebiotic effect on the human gut microbiota: a double-blind, placebo-controlled, crossover study. Br. J. Nutr. *99*, 110-120.

Cruz-Correa, M., and Giardiello, F.M. (2002). Diagnosis and management of hereditary colon cancer. Gastroenterol. Clin. N. Am. *31*, 537-549.

Cummings, J.H. (1984). Constipation, dietary fiber and the control of large bowel function. Postgrad. Med. J. *60*, 811-819.

de Jong, M.M., Nolte, I.M., te Meerman, G.J., van der Graaf, W.T., de Varies, E.G., Fijmons, R.H., Hofstra, R.M., and Kleibeuker, J.H. (2002). Low-penetrance genes and their involvement in colorectal cancer susceptibility. Cancer Epidemiol. Biomark Prev. *11*, 1332-1352.

Dixit, R., Teel, R.W., Daniel, F.B., and Stoner, G.D. (1985). Inhibition of benzo[a]pyrene and benzo[a]pyrene-trans-7, 8-diol metabolism and DNA binding in mouse lung explants by ellagic acid. Cancer Res. *45*, 2951-2956.

Dixit, R., and Gold, B. (1986). Inhibition of *N*-methyl-*N*-nitrosourea-induced mutagenicity and DNA methylation by ellagic acid. Proc. Natl. Acad. Sci. USA *83*, 8039-8043.

Djordjevic, T.M., Siler-Marinkovic, S.S., and Dimitrijevic-Brankovic, S.I. (2011). Antioxidant activity and total phenolic content in some cereals and legumes. Int. J. Food Prop. *14*, 175-184.

Dobashi, Y. (2005). Cell cycle regulation and its aberrations in human lung carcinoma. Pathol. Int. 55, 95-105.

Drankhan, K., Carter, J., Madl, R., Klopfenstein, C., Padula, F., Lu, Y., Warren, T., Schmitz, N., and Takemoto, D.J. (2003). Antitumor activity of wheats with high orthophenolic content. Nutrition and Cancer *47*, 188-194.

DuBois, R.N., Awad, J., Morrow, J., Robert, L.J. 2nd, and Bishop, P.R. (1994). Regulation of eicosanoid production and mitogenesis in rat intestinal epithelial cells by transforming growth factor-alpha and phorbol ester. J. Clin. Invest. *93*, 493-498.

Eberhardt, M.V., Lee, C.Y., and Liu, R.H. (2000). Antioxidant activity of fresh apples. Nature *405*, 903-904.

Eberhart, C.E., Coffery, R.J., Radhika, A., Giardiello, F.M., Ferrenbach, S., and DuBois, R.N. (1994). Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. Gastroenterology *107*, 1183-1188.

ECD. (2008). European Commission Directive. 2008/100/EC of 28 October amending Council Directive 90/496/EEC on nutrition labeling for foodstuffs as regards recommended daily allowance, energy conversion factors and definitions. Oj L 285/9 29.10.2008. Available from: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2008:285:0009:0012:EN:PDF

Edelmann W., Cohen, P.E., Kane, M., Morrow, B., Bennett, S., Umar, A., Kunkel, T., Cattoretti, G., Chaganti, R., Pollard, J.W., Kolodner, R.D., and Kucherlapati, R. (1996). Meiotic pachytene arrest in MLH1-deficient mice. Cell *85*, 125-1134.

Edelmann, W., Tang, K., Umar, A., Heyer, J., Lau, K., Fan, K., Liedtke, W., Cohen, P.E., Kane, M.F., Lipford, J.R., Yu, N., Crouse, G.F., Pollard, J.W. Kunkel, T., Lipkin, M., Kolodner, R., and Kucherlapati, R. (1997). Mutation in the mismatch repair gene Msh6 causes cancer susceptibility. Cell *91*, 467-477.

Espín J.C., González-Barrio, R., Cerdá, B., López-Bote, C., Rey, A.I., and Tomás-Barberán, F.A. (2007). Iberian Pig as a model to clarify obscure points in the bioavailability and metabolism of ellagitannins in humans. J. Agric. Food Chem. *55*, 10476-10485.

Faried, A., Kurnia, D., Faried, L.S., Usman, N., Miyazaki, T., Kato, H., and Kuwano, H. (2007). Anticancer effects of gallic acid isolated from Indonesian herbal medicine,

Phaleria macrocarpa (Scheff.) Boerl, on human cancer cell lines. Int. J. Oncol. *30*, 605-613.

Fearnhead, N.S., Britton, M.P., and Bodmer, W.F. (2001). The ABC of APC. Hum. Mol. Genet. *10*, 721-733.

Fearon, E.R., and Vogelstein, B. (1990). A genetic model for colorectal tumorigenesis. Cell *61*, 759-767.

Fearon, E.R., Cho, K.R., Nigro, J.M., Kern, S.E., Simons, J.W., Ruppert, J.M., Hamilton, S.R., Preisinger, A.C., Thomas, G., Kinzler, K.W., and Vogelstein, B. (1990). Identification of a chromosome 18q gene that is altered on colorectal cancers. Science *247*, 49-56.

Fjaeraa, C., and Nånberg, E. (2009). Effect of ellagic acid on proliferation, cell adhesion and apoptosis in SH-SY5Y human neuroblastoma cells. Biomed. Pharmacother. *63*, 254-261.

Fleming, S.E., Marthinsen, D., and Kuhnlein, H. (1983). Colonic function and fermentation in men consuming high fiber diets. J. Nutr. *113***,** 2535-1544.

Forrester, K., Almoguera, C., Han, K., Grizzle, W.E., and Perucho, M. (1987). Detection of K-ras oncogenes during human colon tumorigenesis. Nature *327*, 298-303.

Gallardo, C., Jimenez, L., and Garcia-Conesa, M.T. (2006). Hydroxycinnamic acid composition and in vitro antioxidant activity of selected grain fractions. Food Chem. 99, 455-463.

Gallo, O., Masini, E., Morbidelli, L., Franchi, A., Fini-Storchi, I., Vergari, W.A., and Ziche, M. (1998). Role of nitric oxide in angiogenesis and tumor progression in head and neck cancer. J. Natl. Cancer Inst. *90*, 587-596.

Garcia-Cardena, G., Oh, P., Liu, J., Schnitzer, J.E., and Sessa, W.C. (1996). Targeting of nitric oxide synthase to endothelial cell caveolae via palmitonylation: implications for nitric oxide signalling. Proc. Natl. Acad. Sci. USA 93, 6448-6553.

Garrison, A.P., Helmrath, M.A., and Dekaney, C.M. (2009). Intestinal stem cells. J. Pediatr. Gastroenterol. Nutr. *49*, 2-7.

Gazzaniga, J.M., and Lupton, J.R. (1987). Dilution effect of dietary fiber sources: an in vivo study in the rat. Nutr. Res. *7*, 1261-1266.

Geller, D.A., and Billiar, T.R. (1998). Molecular biology of nitric oxide synthases. Cancer Metast. Rev. *17*, 7-23.

Giardiello, F.M., Hamilton, S.R., Krush, A.J., Piantadosi, S., Hylind, L.M., Celano, P., Booker, S.V., Robinson, C.R., and Offerhaus, G.J. (1993). Treatment of colonic and

rectal adenomas with sulindac in familial adenomatous polyposis. New Engl. J. Med. *328*, 1313-1316.

Giovannucci, E., Stampfer, M.J., Colditz, G., Rimm, E.B., and Willett, W.C. (1992). Relationship of diet to risk of colorectal adenoma in men. J. Natl. Cancer Inst. *84*, 91-98.

Giovannucci, E., Rimm, E.B., Stampfer, M.J., Colditz, G.A., Ascherio, A., and Willett, W.C. (1994). Aspirin use and the risk of colorectal cancer and adenoma in male health professionals. Ann. Intem. Med. *121*, 241-246.

Giovannucci, E. Egan, K.M., Hunter, D.J., Stampfer, M.J., Colditz, G.A.,Willett, W.C., and Speizer, F.E. (1995). Aspirin and the risk of colorectal cancer in women. N. Engl. J. Med. *333*, 609-614.

Giovannucci, E. (2002). Modifiable risk factors for colon cancer. Gastroenterol. Clin. North Am. *31*, 925-943.

Giovannucci, E., and Wu, K. (2006). Cancers of the colon and rectum. In Cancer Epidemiology and Prevention, D. Schottenfeld, J. Fraumeni, eds. (New York: Oxford University Press), pp. 809-829.

Gonthier, M.P., Verny, M.A., Besson, C., Remesy, C., and Scalbert, A. (2003). Chlorogenic acid bioavailability largely depends on its metabolism by the gut microflora in rats. J. Nutr. *133*, 1853-1859.

Grady, W.M. (2003). Genetic testing for high risk colon cancer patients. Gastroenterology *124*, 1574-1594.

Grady, W.M. (2004). Genomic instability and colon cancer. Cancer Metast. Rev. *23*, 11-27.

Graham, D., Campen, D., Hui, R., Spence, M., Cheetham, C., Levy, G., Shoor, S., and Ray, W. (2005). Risk of acute myocardial infarction and sudden cardiac death in patients treated with cyclo-oxygenase 2 selective and non selective non-steroidal anti-inflammatory drugs: nested case-control study. Lancet *365*, 475-481.

Green, D., and Reed, J.C. (1998). Mitochondria and apoptosis. Science *281*, 1309-1312.

Green, J.E., and Hudson, T. (2005). The promise of genetically engineered mice for cancer prevention studies. Nat. Rev. Cancer *5*, 184-198.

Gupta, R.A., and DuBois, R.N. (2001). Colorectal cancer prevention and treatment by inhibition of cyclooxygenase-2. Nat. Rev. Cancer *1*: 11-21.

Ha, M.A., Jarvis, M.C., and Mann, J.I. (2000). A definition for dietary fiber. Eur. J. Clin. Nutr. *54*, 861-864.

Hague, A., Moorghen, M., Hicks, D., Champman, M., and Paraskeva, C. (1994). Bcl-2 expression in human colorectal adenomas and carcinomas. Oncogene *9*, 3367-3370.

Hall, P.A., Coates, P.J., Ansari, B., and Hopwood, D. (1994). Regulation of cell number in the mammalian gastrointestinal tract: the importance of apoptosis. J. Cell Sci. *107*, 3569-3577.

Hamilton, S.R., Stephens, R.B., Natuzzi, E., Boitnott, J.K., and Yardley, J.H. (1982). Morphologic analogy of intestinal tract carcinogenesis in adenomatous polyposis and the azoxymethane-treated rat model. Lab Invest. *46*, 33A-34A.

Han, E.K.-H., Ng, S.-C., Arber, N., Begemann, M., and Weinstein, I.B. (1999). Roles of cyclin D1 and related genes in growth inhibition, senescence and apoptosis. Apoptosis *4*, 213-219.

Harris, P.J., and Ferguson, L.R. (1993). Dietary fiber: Its composition and role in protection against colorectal cancer. Mutat. Res. *290*, 97-110.

Hawk, E.T., Umar, A., Richmond, E., and Viner, J.I. (2005). Prevention and therapy of colorectal cancer. Med. Clin. N. Am. *89*, 85-110.

He, T.C., Sparks, A.B., Rago, C., Hermeking, H., Zawel, L., da Costa, L.T., Morin, P.J., Vogelstein, B., and Kinzler, K.W. (1998). Identification of c-Myc as a target of the APC pathway. Science *281*, 1509-1512.

Heavey, P.M., McKenna, D., and Rowland, I.R. (2004). Colorectal cancer and the relationship between genes and the environment. Nutrition and Cancer *48*, 124-141.

Heinen, C.D., Richardson, D., White, R., and Groden, J. (1995). Miscrosatellite instability in colorectal adenocarcinoma cell lines that have full-length adenomatous polyposis coli protein. Cancer Res. *55*, 4797-4799.

Herrmann, K. (1989). Occurrence and content of hydroxycinnamic acid and hydroxybenzoic acid compounds in foods. Crit. Rev. Food Sci. Nutr. *28*, 315-247.

Hiltunen, M.O., Alhonen, L., Koitinaho, J., Myöhänen, S., Pääkkönen, M., Marin, S., Kosma, V.M., and Jänne, J. (1997). Hypermethylation of the APC (adenomatous polyposis coli) gene promoter region in human colorectal carcinoma. Int. J. Cancer *70*, 644-648.

Houlston, R.S., Collins, A., Slack, J., and Morton, N.E. (1992). Dominant genes for colorectal cancer are not rare. Ann. Hum. Genet. *56*, 99-103.

Howe, L.R., Subbaramaiah, K., Brown, A.M., and Dannenberg, A.J. (2001). Cyclooxygenase-2: a target for the prevention and treatment of breast cancer. Endocr. Relat. Cancer *8*, 97-114.

Hsi, L.C., Angerman-Stewart, J., and Eling, T.E. (1999). Introduction of full-length APC modulates cyclooxygenase-2 expression in HT-29 human colorectal carcinoma cells at the translational level. Carcinogenesis *20*, 2045-2049.

Hsu, W., Zeng, L., and Costantini, F. (1999). Identification of a domain of Axin that binds to the serine/threonine protein phosphatase 2A and a self-binding domain. J. Biol. Chem. *274*, 3439-3445.

Huang, M.-T., Smart, R.C., Wong, C-Q., and Conney, A.H. (1988). Inhibitory effect of curcumin, chlorogenic acid, caffeic acid and ferulic acid on tumor promotion in mouse skin by 12-O-tetradecanoylphorbol-13-acetate. Cancer Res. *48*, 5941-5946.

Huerta, S., Goulet, E.J., and Livingston, E.H. (2006). Colon cancer and apoptosis. Am. J. Surg. *191*, 517-526.

Ilyas, M. (1997). B-catenin mutations in cell lines established from human colorectal cancers. Proc. Natl. Acad. Sci. USA *99*, 2254-2259.

Imai, K., and Yamamoto, H. (2008). Carcinogenesis and microsatellite instability: the interrelationship between genetics and epigenetics. Carcinogenesis *29*, 673-680.

Inoue, M., Sakaguchi, N., Isuzugawa, K., Tani, H., and Ogihara, Y. (2000). Role of reactive oxygen species in gallic acid-induced apoptosis. Biol. Pharm. Bull *23*, 1153-1157.

Itoh, F., Hinoda, Y., Ohe, M., Ohe, Y., Ban, T., Endo, T., Imais, K., and Yachi, A. (1993). Decreased expression of DCC mRNA in human colorectal cancers. Int. J. Cancer *53*, 260-263.

Jacoby, R.F., Lior, X., Teng, B.B., Davidson, N.O., and Brasitus, T.A. (1991). Mutations in the K-ras oncogene induced by 1,2-dimethylhydrazine in the preneoplastic and neoplastic rat colonic mucosa. J. Clin. Invest. *87*, 624-630.

Jaiswal, A.S., Balusu, R., and Narayan, S. (2005). Involvement of adenomatous polyposis coli in colorectal tumorigenesis. Front. Biosci. *10*, 1118-1134.

Jaiswal, M., LaRusso, N., Burgart, L., and Gores, G. (2000). Inflammatory cytokines induce DNA damage and inhibit DNA repair in colonic carcinoma cells by a nitric oxide-dependent mechanism. Cancer Res. *60*, 184-189.

James, S.L., Muir, J.G., Curtis, S.L., and Gibson, P.R. (2003). Dietary fibre: a roughage guide. Intern. Med. J. *33*, 291-296.

Jiang, Y., Kusama, K., Satoh, K., Takayama, E., Watanabe, S., and Sakagami, H. (2000). Induction of cytotoxicity by chlorogenic acid in human oral tumor cell lines. Phytomedicine *7*, 483-491.

Johnson, I.T. (2007). Gut health, genetics and personalized nutrition. Genes Nutr. *2*, 53-54.

Jones, B.A., and Gores, G.J. (1997). Physiology and pathophysiology of apoptosis in epithelial cells of the liver, pancreas, and initestine. Am. J. Pathol. *273*, G1174-G1188.

Kargman, S.L., O'Neil, G.P., Vickers, P.J., Evans, J.F., Mancini, J.A., and Jothy, S. (1995). Expression of prostaglandin G/H synthase-1 and -2 proteins in human colon cancer. Cancer Res. *55*, 2556-2559.

Kashiwagi, H., and Spigelman, A.D. (2000). Gastroduodenal lesions in familial adenomatous polyposis. Surg. Today *30*, 675-682.

Kato J., Matsushime, H., Hiebert, S.W., Ewan, M.E., and Sherr, C.J. (1993). Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclin D-dependent kinase CDK4. Genes Dev. *7*, 331-342.

Kato, M., Ito, Y., Kobayashi, S., and Isono, K. (1996). Detection of DCC and Ki-ras gene alterations in colorectal carcinoma tissue as prognostic markers for liver metastatic recurrence. Cancer Suppl. *77*, 1729-1735.

Kawamori, T., Takahashi, M., Watanabe, K., Ohta, T., Nakatsuqi, S., Suqimura, T., and Wakabayashi, K. (2000). Suppression of azoxymethane-induced colonic aberrant crypt foci by a nitric oxide synthase inhibitor. Cancer Lett. *148*, 33-37.

Kazanov, D., Shapira, I., Pick, M., Kolker, O., Liberman, E., Deutsch, V., Strier, L., Dvory-Sobol, H., Kunik, T., and Arber, N. (2003). Oncogenic transformation of normal enterocytes by overexpression of cyclin D1. Digest. Dis. Sci. *48*, 1251-1261.

Kerr, J.F.R., Wyllie, A.H., and Currie, A.R. (1972). Apoptosis: a basic biological phenomenon with wide ranging implications in tissue kinetics. Br. J. Cancer *26*, 239-257.

Kim, K.-H., Tsao, R., Yang R., and Cui, S.W. (2006). Phenolic acid profiles and antioxidant activities of wheat bran extracts and the effect of hydrolysis conditions. Food Chem. *95***,** 466-473.

Kim, P.K., Zamora, R., Petrosko, P., and Billiar, T.R. (2001). The regulatory role of nitric oxide in apoptosis. Int. Immunopharmacol. *1*, 1421-1441.

Kinzler, K.W., Nilbert, M.C., Su, L-K., Vogelstein, B., Bryan, T.M., Levy, D.B., Smith, K.J., Preisinger, A.C., Hedge, P., McKechnie, D., Finniear, R., Markham, A., Groffen, J., Boguski, M.S., Altschui, S.F., Horji, A., Ando, H., Miyoshi, Y., Miki, Y., Nishisho, I., and Nakamura, Y. (1991). Identification of FAP locus genes from chromosome 5q21. Science *253*, 661-665.

Klepacka, J., and Fornal, L. (2006). Ferulic acid and its position among the phenolic componds of wheat. Crit. Rev. Food Sci. *46***,** 639-647.

Knudson, A.G. (2001). Two genetic hits (more or less) to cancer. Nat. Rev. Cancer *1*, 157-162.

Kolodner, R. (1996). Biochemistry and genetics of eukaryotic mismatch repair. Genes Dev. *10*, 1433-1442.

Krajewski, S., Krajewska, M., Shabaik, A., Miyashita, T., Wang, H.G., and Reed, J.C. (1994). Immunohistochemical determination of in vivo distribution of Bax, a dominant inhibitor of Bcl-2. Am. J. Pathol. *145*, 1323-1336.

Kramer, B., Kramer, W., and Fritz, H.J. (1984). Different base/base mismatches are corrected with different efficiencies by the methyl-directed DNA mismatch-repair system of *E. coli*. Cell *38*, 879-887.

Kritchevsky, D. (1997). Dietary fibre and cancer. Eur. J. Cancer Prev. *6*, 435-441.

Kritchevsky, D. (1999). Protective role of wheat bran fiber: preclinical data. Am. J. Med. *25*, 28S-31S.

Kutchera, W., Jones, D.A., Matsunami, N., Groden, J., Mcintyre, T.M., Zimmerman, G.A., White, R.L., and Prescott, S.M. (1996). Prostaglandin H synthase 2 is expressed abnormally in human colon cacner: evidence for a transcriptional affect. Proc. Natl. Acad. Sci. USA *93*, 4816-4820.

Kune, G.A., Kune, S., and Watson, L.F. (1988). Colorectal cancer risk, chronic illnesses, operations, and medications: case control results from the Melbourne Colorectal Cancer Study. Cancer Res. *48*, 4399-4404.

Ladenheim, J., Garcia, G., Titzer, D., Herzenberg, H., Lavori, P., Edson, R., and Omary, M.B. (1995). Effect of sulindac on sporadic colonic polyps. Gastroenterology *108*, 1083-1087.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*, 680-685.

Larrosa, M., Tomás-Barberán, F.A., and Espín J.C. (2006). The dietary hydrolysable tannin punicalagin releases ellagic acid that induces apoptosis in human colon adenocarcinoma Caco-2 cells by using the mitochondrial pathway. J. Nutr. Biochem. *17*, 611-625.

Larsson, S.C., Bergkvist, L., Rutegard, J., Giovannucci, E., and Wolk, A. (2006). Calcium and dairy food intakes are inversely associated with colorectal cancer risk in the Cohort of Swedish Men. Am. J. Clin. Nutr. *73*, 1-2.

Laurent-Puig, P., Beroud, C., and Soussi, T. (1998). APC gene: database of germline and somatic mutations in human tumors and cell lines. Nucleic Acids Res. *26*, 269-270.

Le Leu, R.K., Brown, I.L., Hu, Y., Morita, T., Esterman, A., and Young, G.P. (2006). Effect of dietary resistant starch and protein on colonic fermentation and intestinal tumourigenesis in rats. Carcinogenesis *28*, 240-245.

Lesca, P. (1983). Protective effects of ellagic acid and other plant phenols on benzo[a]pyrene-induced neoplasia in mice. Carcinegenesis *4*, 1651-1653.

Li, T.M., Chen, S.W., Su, C.C., Ling, J.G., Yeh, C.C., Cheng, K.C., and Chung, J.G. (2005). Ellagic acid induced p53/p21 expression, G1 arrest and apoptosis in human bladder cancer T24 cells. Anticancer Res. *25*, 971-979.

Lichtenstein, D.R., Syngal, S., and Wolfe, M.M. (1995). Nonsteroidal antiinflammatory drugs and the gastrointestinal tract. The double-edged sword. Arthritis Rheum. *38*, 5-18.

Lipkin, M. (1973). Proliferation and differentiation of gastrointestinal cells. Physiol. Rev. *53*, 891-915.

Lipkin, M. (1985). Growth and development of gastrointestinal cells. Annu. Rev. Physiol. *47*, 175-197.

Liu, M.C., and Gelmann, E.P. (2002). P53 gene mutations: case study a clinical marker for solid tumors. Semin. Oncol. *29*, 246-257.

Liu, R.H., and Hotchkiss, J.H. (1995). Potential genotoxicity of chronically elevated nitric oxide: a review. Mutat. Res. *339*, 73-89.

Liu, R.H. (2004). Potential synergy of phytochemicals in cancer prevention: mechanism of action. J. Nutr. *134*, 3479S-3485S.

Liu, X.-H., and Rose, D.P. (1996). Differential expression and regulation of cyclooxygenase-1 and -2 in two human breast cancer cell lines cancer. Res. *56*, 5125-5127.

Liyana-Pathirana, C.M., and Shahidi, F. (2006). Importance of insoluble-bound phenolics to antioxidants properties of wheat. J. Biol. Chem. *54*, 1256-1264.

Loraine, M., De, P., Dryer, D., Ellison, L., Grunfeld, E., Logan, H., MacIntyre, M., Mery, L., Morrison, H., and Weir, H.K. (2009). Canadian Cancer Statistics. Toronto : Canadian Cancer Society.

Losi, L., Roncucci, L., di Gregorio, C., de Leon, M.P., and Benhattar, J. (1996). K-ras and p53 mutations in human colorectal aberrant crypt foci. J. Pathol. *178*, 259-163.

Losso, J.N., Bansode, R.R., Trappey, A. 2nd, Bawadi, H.A., and Truax, R. (2004). In vitro anti-proliferative activities of ellagic acid. J. Nutr. Biochem. *15*, 672-678.

Lowsky, R., DeCoteau, J.F., Reitmair, A.H., Ichinohasama, R., Dong, W.F., Mak, T.W., Kadin, M.E. and Minden, M.D. (1997). Defects of the mismatch repair gene MSH2

are implicated in the development of murine and human lymphoblastic lymphomas and are associated with the aberrant expression of rhombotin-2 (Lmo-2) and Tal- 1 (SCL). Blood *89*, 2276-2282.

Lowy, D.R., and Willumsen, B.M. (1993). Function and regulation of ras. Annu. Rev. Biochem. *62*, 851-891.

Luebeck, E.G., and Moolgavkar, S.H. (2002). Multistage carcinogenesis and the incidence of colorectal cancer. Proc. Natl. Acad. Sci. USA *99*, 15095-15100.

Lupton, J.R., and Meacher, M.M. (1988). Radiographic analysis of the effect of dietary fibers on rat colonic transit time. Am. J. Physiol. *255*, G633-G639.

Lupton, J.R., and Turner, N.D. (1999). Potential protective mechanisms of wheat bran fiber. Am. J. Med. *106*, 24S-27S.

Lynch, H.T., Shaw, M.W., Magnuson, C.W., Larsen, A.L., and Krush, A.J. (1966). Hereditary factors in two large Midwestern kindreds. Arch. Intern. Med. *117*, 206-212.

Lynch, H.T., Smyrk, T.C., Watson, P., Lanspa, S.J., Lynch, J.F., Lynch, P.M., Cavalieri, R.J., and Boland, C.R. (1993). Genetics, natural hisory, tumor spectrum, and pathology of heriditary nonpolyposis colorectal cancer : an updated review. Gastroenterology *104*, 1535-1549.

Lynch, H.T., and de la Chapelle, A. (2003). Hereditary colorectal cancer. N. Engl. J. Med. *348*, 919-932.

Macfarlane, G.T., Gibson, G.R., and Cummings, J.H. (1992). Comparison of fermentation reactions in different regions of the human colon. J. Appl. Bacteriol. *72*, 57-64.

Mahmoud, N.N., Carothers, A.M., Grunberger, D., Bilinski, R.T., Churchill, M.R., Martucci, C., Newmark, H.L., and Bertagnolli, M.M. (2000). Plant phenolics decrease intestinal tumors in an animal model of familial adenomatous polyposis. Carcinogenesis *21*, 921-927.

Malhotra, S., Shafiq, N., and Pandhi, P. (2004). COX-2 inhibitors: a class act or just vigourously promoted. Med. Gen. Med. *6*, 6.

Malik, A., Afaq, F., Safaraz, S., Adhami, V.M., Syed, D.N., and Mukhtar, H. (2005). Pomegranate fruit juice for chemopreventon and chemotherapy of prostate cancer. Proc. Natl. Acad. Sci. USA *102*, 14813-14818.

Mandal, M., Adam, L., Mendelsohn, J., and Kumar, R. (1998). Nuclear targeting of Bax during apoptosis in human colorectal cancer cells. Oncogene *17*, 999-1007.

Mandal, S., and Stoner, G.D. (1990). Inhibition of N-nitroso-benzylmethylamine-induced esophageal tumorigenesis in rats by ellagic acid. Carcinegesis. *11*, 55-61.

Marteau, P. (2006). Probiotic, prebiotic, synbiotics: ecological treatment for inflammatory bowel disease? Gut *55*, 1692-1693.

Mazelin, L., Bernet, A., Bonod-Bidaud, C., Pays, L., Arnaud, S., Gespach, C., Bredesen, D.E., Scoazec, J.Y., and Mehlen, P. (2004). Netrin-1 controls colorectal tumorigenesis by regulating apoptosis. Nature *431*, 80-84.

Maziya-Dixon, B., Klopfenstein, C., and Leipold, H.W. (1994). Protective effects of hard red versus hard white winter wheats in chemically induced colon cancer in CFI mice. Cereal Chem. *71*, 359-363.

McCann, M.J., Gill, C.I.R., O'Brien, G., Rao, J.R., McRoberts, W.C., Hughes, P., McEntee, R., and Rowland, I.R. (2007). Anti-cancer properties of phenolics from apple waste on colon carcinogenesis in vitro. Food Chem. Toxicol. *45*, 1224-1230.

McDonnell, T.J. (1993). Cell division versus cell death: a functional model of multistep neoplasia. Mol. Carcinog. *8*, 209-213.

McIntyre, A., Gibson, P.R., and Young, G.P. (1993). Butyrate production from dietary fiber and protection against large bowel cancer in rat model. Gut *34*, 386-391.

Mecklin, J.P. (1987). Frequency of hereditary colorectal carcinoma. Gastroenterology *93*, 1021-1025.

Medh, R.D., and Thompson, E.B. (2000). Hormonal regulation of physiological cell turnover and apoptosis. Cell Tissue Res. *301*, 101-124.

Mei, J.M., Hord, N.G., Winterstein, D.F., Donald, S.P., and Phang, J.M. (2000). Expression of prostaglandins endoperoxide H synthase-2 by nitric oxide in conditionally immortalized murine colonic epithelial cells. FASEB J. *14*, 1188-1192.

Merritt, A.J., Potten, C.S., Kemp, C.J., Hickman, J.A., Ballmain, A., Lane, D.P., and Hall, P.A. (1994). The role of p53 in spontaneous and radiation-induced apoptosis in the gastrointestinal tract of normal and p53-deficient mice. Cancer Res. 54, 614-617.

Merritt, A.J., Potten, C.S., Watson, A.J., Loh, D.Y., Nakayama, K., and Hickman, J.A. (1995). Differential expression of bcl-2 in intestinal epithelia. Correlation with attenuation of apoptosis in colonic crypts and the incidence of colonic neoplasia. J. Cell Sci. *108*, 2261-2271.

Mertens-Talcott, S.U., Bomser, J.A., Romero, C., Talcott, S., and Percival, S.S. (2005). Ellagic acid potentiates the effect of quercetin on p21waf1/cip1, p53, and MAP-kinases without affecting intracellular generation of reactive oxygen species in vitro. J. Nutr. *135*, 609-614.

Midgley, C.A., White, S., Howitt, R., Save, V., Dunlop, M.G., Hall, P.A., Lane, D.P., Wyllie, A.H., and Bubb, V.J. (1997). APC expression in normal human tissues. J. Pathol. *181*, 426-433.

Mirvish, S.S., Cardesa, A., Wallcave, L., and Shubik, P. (1975). Induction of mouse lung adenomas by amines or ureas plus nitrite and by N-nitroso compounds: effect of ascorbate, gallic acid, thiocyanate, and caffeine. J. Natl. Cancer Inst. *55*, 633-636.

Miyoshi, Y., Nagase, H., Ando, H., Horii, S., Nakatsuru, S., Aoki, T., Miki, Y., Mori, T., and Nakamura, Y. (1992). Somatic mutations of the APC gene in colorectal tumors: mutation cluster regionin the APC gene. Hum. Mol. Genet. *1*, 229-233.

Mohamad, N., Gutiérrez, A., Núñez, M., Cocca, C., Martín, G., Cricco, G., Medina, V., Rivera, E., and Bergoc, R. (2005). Mitochondrial apoptotic pathways. Biocell *29*, 149-161.

Moncada, S., Palmer, R.M.J., and Higgs, E.A. (1991). Nitrix oxide: physiology, pathophysiology, and pharmacology. Pharmacol. Rev. *43*, 109-142.

Moon, R.T., Kohn, A.D., De Ferrari, G.V., and Kaykas, A. (2004). Ant and β-catenin signalling: diseases and therapies. Nat. Rev. Genet. *5*, 689-699.

Morin, P.J., Vogelstein, B, and Kinzler, K.W. (1996). Apoptosis and APC in colorectal tumorigenesis. Proc. Natl. Acad. Sci. USA *93*, 7950-7954.

Morin, P.J., Sparks, A.B., Korinek, V., Barker, N., Clevers, H., and Vogelstein, B. (1997). Activation of β-catenin-Tcf signalling in colon cancer by mutations in β-catenin or APC. Science *275*, 1787-1790.

Mukhtar, H., Das, M., Del Tito, B., and Bickers, D. (1984). Protection against 3-methylcholanthrene-induced skin tumorigenesis in BALB/c mice by ellagic acid. Biochem. Biophys. Res. Commun. *119*, 751-757.

Munemitsu, S., Albert, I., Souza, B., Rubinfeld, B., and Polakis, P. (1995). Regulation of intracellular β-catenin levels by the adenomatous polyposis coli (APC) tumor-suppressor protein. Proc. Natl Acad. Sci. USA *92*, 3046–3050.

Nakane, M., Schmidt, H.H.H.W., Pollock, J.S., Forstermann, U., and Murad, F. (1993). Cloned human brain nitric oxide synthase is highly expressed in skeletal muscle. FEBS Lett. *316*, 175-180.

Nakayama, K., Negishi, I., Kuida, K., Shinkai, Y., Louie, M.C., Fields, L.E., Lucas, P.J., Stwart, V., Alt, F.W., and Loh, D.Y. (1993). Disappearance of the lymphoid system in Bcl-2 homozygous mutant chimeric mice. Science *261*, 1584-1588.

Nakayama, K., Negishi, I., Kuida, K., Sawa, H., and Loh, D.Y. (1994). Targeted disruption of Bcl-2 alpha beta in mice: occurrence of gray hair, polycystic kidney disease, and lymphocytopenia. Proc. Natl. Acad. Sci. USA *91*, 3700-3704.

Narayanan, B.A., Geoffroy, O., Willingham, M.C., Re, G.G., and Nixon, D.W. (1999). P53/p21 (WAF1/CIP1) expression and its possible role in G1 arrest and apoptosis in ellagic acid treated cancer cells. Cancer Lett. *136*, 215-221.

Natella, F., Nardini, M., Di Felice, M., and Scaccini, C. (1999). Benzoic and cinnamic acid derivatives as antioxidants: structure-activity relation. J. Agric. Chem. *47*, 1453-1459.

Nguyen, T., Brunson, D., Crespi, C.L., Penman, B.W., Wishnok, J.S., and Tannenbaum, S.R. (1992). DNA damage and mutation in human cells expose to nitric oxide in vivo. Proc. Natl. Acad. Sci. USA. *89*, 3030-3034.

Nigro, N.D., Bull, A.W., Klopfer, B.A., Pak, M.S., and Campbell, R.L. (1979). Effect of the dietary fiber on azoxymethane-induced intestinal carcinogenesis in rats. J. Natl. Cancer Inst. *63*, 1097-1102.

Norat, T., Bingham, S., Ferrari, P., Slimani, N., Jenab, M., Mazuir, M., Overvad, K., Olsen, A., Tjønneland, A., Clavel, F., Boutron-Ruault, M.C., Kesse, E., Boeing, H., Bergmann, M.M., Nieters, A., Linseisen, J., Trichopoulou, A., Trichopoulos, D., Tountas, Y., Berrino, F., Palli, D., Panico, S., Tumino, R., Vineis, P., Bueno-de-Mesquita, H.B., Peeters, P.H., Engeset, D., Lund, E., Skeie, G., Ardanaz, E., González, C., Navarro, C., Quirós, J.R., Sanchez, M.J., Berglund, G., Mattisson, I., Hallmans, G., Palmqvist, R., Day, N.E., Khaw, K.T., Key, T.J., San Joaquin, M., Hémon, B., Saracci, R., Kaaks, R., and Riboli, E. (2005). Meat, fish, and colorectal cancer risk: the European Prospective Investigation into cancer and nutrition. J. Natl. Cancer Inst. *97*, 906-916.

Norat, T., and Riboli, E. (2003). Dairy products and colorectal cancer. A review of possible mechanisms and epidemiological evidence. Eur. J. Clin. Nutr. *57*, 1-17.

Ohno, Y., Fukuda, K., Takemura, G., Toyota, M., Watanabe, M., Yasuda, N., Xinbin, Q., Maruyama, R., Akao, S., Gotou, K., Fujiwara, T., and Fujiwara, H. (1999). Induction of apoptosis by gallic acid in lung cancer cells. Anticancer Drugs *10*, 3237-3248.

Onyeneho, N.S., and Hettiarachchy, N.S. (1992). Antioxidant activity of durum wheat bran. J. Agric. Food Chem. *40*, 1496-1500.

Ookawa, K., Sakamoto, M., Hirohashi, S., Yoshida, Y., Sugimura, T., Terada, M., and Yokota, J. (1993). Concordant p53 and DCC alterations and allelic losses on chromosomes 13q and 14q associated with liver metastases of colorectal carcinoma. Int. J. Cancer *53*, 382-387.

Oshima, M., Oshima, H., Kitagawa, K., Kobayashi, M., Itakura, C., and Taketo, M. (1995). Loss of Apc heterozygosity and abnormal tissue building in nascent intestinal polyps in mice carrying a truncated Apc gene. Proc. Natl. Acad. Sci. USA *92*, 4482-4486.

Oshima, M., Dinchuk, J.E., Kargman, S.L., Oshima, H., Hancock, B., Kwong, E., Trzaskos, J.M., Evans, J.F., and Taketo, M.M. (1996). Suppression of intestinal polyposis in Apc delta716 knockout mice by inhibition of cyclooxygenase 2 (COX-2). Cell *87*, 803-809.

Paradisi, A., Maisse, C., Coissieux, M.M., Gadot, N., Lépinasse, F., Delloye-Bourgeois, C., Delcros, F.G., Svrcek, M., Neufert, C., Fléjou, J.F., Scoazec, J.Y., Mehlen, P. (2009). Netrin-1 up-regulation in inflammatory bowel diseases is required for colorectal cancer progression. Proc.natl. Acad. Sci. USA *106*, 17146-17151.

Pardee, A.B. (1989). G1 events and regulation of cell proliferation. Science *246*, 603-608.

Parkin, D.M. (2004). International variation. Oncogene *23*, 6329-6340.

Parkin, D.M., Bray, F., Ferlay, J., and Pisani, P. (2005). Global cancer statistics, 2002. CA Cancer J. Clin. *55*, 74-108.

Parr, A.J., and Bolwell, J.P (2002). Phenols in the plant and in man. The potential for possible nutritional enhancement of the diet by modifying the phenols content or profile. J. Sci. Food Agric. *80*, 985-1012.

Pegg, A.E., and Byers, T.L. (1992). Repair of DNA containing O^6-alkylguanine. FASEB J. *6*, 2302-2310.

Peltomaki, P., Lothe, R.A., Aaltonen, L.A., Pylkkänen, L., Nyström-Lahti, M., Seruca, R., David, L., Holm, R., Ryberg, D., Haugen, A., Brøgger, A., Børresen, A.-L., and de la Chapelle, A. (1993). Microsatellite instability is associated with tumors that characterize the hereditary nonpolyposis colorectal carcinoma syndrome. Cancer Res. *53*, 5853-5855.

Percesepe, A., Borghi, F., Menigatti, M., Losi, L., Foroni, M., Di Gregorio, C., Rossi, G., Pedroni, M., Sala, E., Vaccina, F., Roncucci, L., Benatti, P., Viel, A., Genuardi, M., Marra, G., Kristo, P., Peltomäki, P., and Ponz de Leon, M. (2001). Molecular screening for hereditary nonpolyposis colorectal cancer: a prospective, population-based study. J. Clin. Oncol. *19*, 3944-3950.

Pereira, D.M., Valentão, P., Pereira, J.A., and Andradem P.B. (2009). Phenolics: from chemistry to biology. Molecules *14*, 2202-2211.

Polakis, P. (1997). The adenomatous polyposis coli (APC) tumor suppressor. Biochim. Biophys. Acta. *1332*, F127-147.

Potten, C.S., Booth, C., and Pritchard, D.M. (1997). The intestinal epithelial stem cell: the mucosal governor. Int. J. Exp. Pathol. *78*, 219-243.

Powell, S.M., Zilz, N., Beazer-Barclay, Y., Bryan, T.M., Hamilton, S.R., Thibodeau, S.N., Vogelstein, B., and Kinzler, K.W. (1992). APC mutations occur early during colorectal tumorigenesis. Nature *359*, 235-237.

Prescott, S.M., and Fitzpatrick, F.A. (2000). Cyclooxygenase-2 and carcinogenesis. Biochim. Biophys. Acta. *1470*, M69–M78.

Pritchard, D.M., Potten, C.S., Korsmeyer, S.J., Roberts, S.A., and Hickman, J.A. (1999). Damage-induced apoptosis in intestinal epithelia from bcl-2-null and bax-null mice: investigations of the mechanistic determinants of epithelial apoptosis in vivo. Oncogene *18*, 7287-7293.

Puchalski, R., and Fahl, W. (1990). Expression of recombinant glutathione S-transferase pi, Ya, or Ybl confers to alkylating agents. Proc. Natl. Acad. Sci. USA *87*, 2443-2447.

Rampino, N., Yamamoto, H., Ionov, Y., Li, Y., Sawai, H., Reed, J.C., and Perucho, M. (1997). Somatic fremeshift mutations in the BAX gene in colon cancers of the microsatellite mutator phenotype. Science *275*, 967-969.

Rao, C.V., Kawamori, T., Hamid, R., and Reddy, B.S. (1999). Chemoprevention of colonic aberrant crypt foci by an inducible nitric oxide synthase-selective inhibitor. Carcinogenesis *20*, 641-644.

Rao, C.V., Indranie, C., Simi, B., and Reddy, B.S. (2002). Chemopreventive properties of selective inducible nitric oxide synthase inhibitor in colon carcinogenesis, administered alone or in combination with celecoxib, a selective cyclooygenase-2 inhibitor. Cancer Res. *62*, 165-170.

Rao, C.V. (2004). Nitric oxide signalling in colon cancer chemoprevention. Mutat. Res. *555*, 107-119.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., and Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Biol. Med. *26*, 1231-1237.

Reddy, B.S., Hedges, A.R., Laakso, K., and Wynder, E.L. (1978). Metabolic epidemiology of large bowel cancer. Fecal bulk and constituents of high-risk North American and low-risk Finnish population. Cancer *42*, 2832-2838.

Reed, J.C. (1999). Mechanisms of apoptosis avoidance in cancer. Curr. Opin. Oncogene *17*, 3225-3236.

Reitmair, A.H., Schmits, R., Ewel, A., Bapat, B., Redston, M., Mitri, A., Waterhouse, P., Mittrucker, H.-W., Wakeham, A., Liu, B., Thomason, A., Griesser, H., Gallinger, S.,

Ballhausen, W.G., Fishel, R., and Mak, T.W. (1995). MSH2 deficient mice are viable and susceptible to lymphoid tumors. Nat. Genet. *11*, 64-70.

Ribas, M., Masramon, L., Aiza G, Capellà, G., Miró, R., and Peinado, M.A. (2003). The structural nature of chromosomal instability in colon cancer cells. FASEB J. *17*, 289–291.

Rice-Evans, C.A., Miller, N.J., and Paganga, G. (1996). Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radical Biol. Med. *20*, 933-956.

Robbins, D.H., and Itzkowitz, S.H. (2002). The molecular and genetic basis of colon cancer. Med. Clin. N. Am. *86*, 1467-1495.

Robert, A. (1983). Cytoprotection of the gastrointestinal mucosa. Adv. Intern. Med. *28*, 325-337.

Ruemmele, F.M., Schwartz, S., Seidman, E.G., Dionne, S., Levy, E., and Lentze, M.J. (2003). Butyrate induced Caco-2 cell apoptosis is mediated via the mitochondrial pathway. Gut *52*, 94-100.

Rupnarain, C., Dlamini, Z., Naicker, S., and Bhoola, K. (2004). Colon cancer: genomics and apoptotic events. Biol. Chem. *385*, 449–464.

Saif, M. W., and Chu, E. (2010). Biology of colorectal cancer. Cancer J. *16*, 196-201.

Salovaara, R., Loukola, A., Kristo, P., Kääriäinen, H., Ahtola, H., Eskelinen, M., Härkönen, N., Julkunen, R., Kangas, E., Ojala, S., Tulikoura, J., Valkama, E., Järvinen, H., Mecklin, J.-P., Aaltonen, L.A., and de la Chapelle, A. Population-Based Molecular Detection of Hereditary Nonpolyposis Colorectal Cancer. J. Clin. Oncol. *18*, 2193-2200.

Salucci, M., Stivala, L.A., Maiani, G., Bugianesi, R., and Vannini, V. (2002). Flavonoids uptake and their effect on cell cycle of human colon adenocarcinoma cells (Caco2). Br. J. Cancer *86*, 1645-1651.

Samowitz, W.S., Curtin, K., Lin, H.H., Robertson, M.A., Schaffer, D., Nichols, M., Gruenthal, K., Leppert, M.F., and Slattery, M.L. (2001). The colon cancer burden of genetically defined hereditary nonpolyposis colon cancer. Gastroenterology *121*, 830-838.

Sancar, A. (1995). DNA repair in humans. Annu. Rev. Genet. *29*, 69-105.

Sandler, R.S., Halabi, S., Baron, J.A., Budinger, S., Paskett, S., Kereszter, R. Petrelli, N., Pipas, M., Karp, D.D., Loprinzi, C.L., Steinbach, G., and Schilsky, R. (2003). A randomized trial of aspirin to prevent colorectal adenomas in patient with previous colorectal cancer. N. Eng. J. Med. *348*, 883-890.

Sano, H., Kawahito, Y., Wilder, R.L., Hashiramoto, A., Mukai, S., Asai, K., Kimura, S., Kato, H., Kondo, M., and Hla, T. (1995). Expression of cyclooxygenase-1 and -2 in human colorectal cancer. Cancer Res. *55*, 3785-3789.

Scheppach, W., Bartram, H.P., and Richter, F. (1995). Role of short-chain fatty acids in the prevention of colorectal cancer. Eur. J. Cancer *31A*, 1077-1080.

Scheppach, W., and Weiler, F. (2004). The butyrate story: old wine in new bottles? Curr. Opin Clin. Nutr. Metab. Care *7*, 563-567.

Seeling, J.M., Miller, J.R., Gil, R., Moon, R.T., White, R., and Virshup, D.M. (1999). Regulation of beta-catenin signalling by the B56 subunit of protein phosphotase 2A. Science *283*, 2089-2091.

Senapathy, J.G., Jayanthi, S., Viswanathan, P., Umadevi, P., and Nalini, N. (2011). Effect of gallic acid on xenobiotic metabolizing enzymes in 1,2-dimethyl hydrazine induced colon carcinogenesis in Wistar rats – A chemopreventive approach. Food Chem. Toxicol. *49*, 887-892.

Senda, T., Shimomura, A., and Lizuka-Kogo, A. (2005). Adenomatous polyposis coli (*Apc*) tumor suppressor gene as a multifunctional gene. Anat. Sci. Int. *80*, 121-131.

Shahidi, F., and Wanasundara, P.K. (1992). Phenolic antioxidants. Crit. Rev. Food Sci. Nutr. *32*, 67-103.

Shahrzad, S., Aoyagi, K., Winter, A., Akio, K., and Bitsch, I. (2001). Pharmacokinetics of gallic acid and its relative bioavailabitly from tea in healthy humans. J. Nutr. *131*, 1207-1210.

Shanmugthasan, M., and Jothy, S. (2000). Apoptosis, anoikis and their relevance to the pathobiology of colon cancer. Pathol. Int. **5***0*, 273-279.

Shao, J., Jung, C., Liu, C., and Sheng, H. (2005). Prostaglandin E_2 stimulates the β-catenin/T cell factor-dependent transcription in colon cancer. J. Biol. Chem. *280*, 26565-26572.

Sheng, H., Shao, J., Morrow, J.D., Beauchamp, R.D., and DuBois, R.N. (1998). Modulation of apoptosis and Bcl-2 expression by prostaglandin E_2 in human colon cancer cells. Cancer Res. *58*, 362-366.

Sheng, H., Shao, J., Washington, M.K., and DuBois, R.N. (2001). Prostaglandin E2 increases growth and motility of colorectal carcinoma cells. J. Biol. Chem. *276*, 18075-18081.

Shewry, P.R. (2009) Wheat. J. Exp. Bot. *60*, 1537-1553.

Shibata, H., Toyoma, K., Shioya, H., to, M., Hirota, M., Hasegawa, S., Matsumoto, H., Takano, H., Akiyama, T., Toyoshima, K., Kanamaru, R., Kanegae, Y., Saito, I., Nakamura, Y., Shiba, K., and Noda, T. (1997). Rapid colorectal adenoma formation initiated ny conditional targeting of the Apc gene. Science *278*, 120-123.

Shtutman, M., Zhurinsky, J., Simcha, I., Albaanese, C., D'Amico, M., Pestell, R., and Ben-Ze'ev, A. (1999). The cyclin D1 gene is a target of the that β-catenin/LEF-1 pathway. Proc. Natl. Acad. Sci. USA *96*, 5522-5527.

Sinicrope, F.A., Ruan, S.B., Cleary, K.R., Stephens, L.C., Lee, J.J., and Levin, B. (1995). Bcl-2 and p53 oncoprotein expression during colorectal tumorigenesis. Cancer Res. *55*, 237-241.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., and Klenk, D.C. (1985). Measurement of protein using bicinchoninic acid. Anal. Biochem. *150*, 76-85.

Sonoshita, M., Takaku, K., Sasaki, N., Sugimoto, Y., Ushikubi, F., Narumiya, S., Oshima, M., and Taketo, M. M. (2001). Acceleration of intestinal polyposis through prostaglandin receptor EP2 in Apc716 knockout mice. Nat. Med. *7*, 1048-1051.

Spiller, G.A., Story, J.A., Lodics, T.A., Pollack, M., Monyan, S., Butterfiels, G., and Spiller, M. (2003). Effect of sun-dried raisins on bile acid excretion, intestinal transit time, and fecal weight: a dose-response study. J. Med. Food *6*, 87-91.

Stalikas, C.D. (2007). Extraction, separation, and detection methods for phenolic acids and flavonoids. J. Sep. Sci. *30*, 3268-3295.

Steinbach, G., Lynch, P.M., Phillips, R.K.S., Wallace, M.H. K., Hawk, E.T., Gordon, G.B., Wakabayashi, N., Saunders, B., Shen, Y., Fujimura, T., Su, L.-K., Levin, B., Godio, L., Patterson, S., Rodriguez-Bigas, M.A., Jester, S.L., King, K.L., Schumacher, M. Abbruzzese, J., DuBois, R.N., Hittelman, W.N., Zimmerman, S., Sherman, J.W., and Kelloff, G. (2000). The effect of celecoxib, a cyclooxygenase-2 inhibitor, in familial adenomatous polyposis. N. Eng. J. Med. *342*, 1946-1952.

Stolina, M., Sharma, S., Lin, Y., Dohadwala, M., Gardner, B., Luo, J., Zhu, L., Kronenberg, M., Miller, P.W. Portanova, J., Lee, J.C., and Dubinett, S.M. (2000). Specific inhibition of cyclooxygenase 2 restores antitumor reactivity by altering the balance of IL-10 and IL-12 synthesis. J. Immunol. *164*,361-370.

Strober, W. (1997). Trypan Blue Exclusion Test of Cell Viability. Curr. Protoc. Immunol. A.3B.1–A.3B.2.

Sturm, I., Köhne, C.H., Wolff, G., Petrowsky, H., Hillebrand, T., Hauptmann, S., Lorenz, M., Dörken, B., and Daniel, P.T. (1999). Analysis of the p53/BAX pathway in colorectal cancer: low BAX is a negative prognostic factor in patients with resected liver metastases. J. Clin. Oncol. *17*, 1364-1374.

Su, L.K., Kinzler, K.W., Volgestein, B., Preisinger, A.C., Moser, A.R., Luongo, C., Gould, K.A., and Dove, W.F. (1992). Multiple intestinal neoplasia caused by a mutation in the murine homolog of the APC gene. Science *256*, 668-670.

Su, L.K., Volgestein, B., and Kinzler, K.W. (1993). Association of the APC tumor suppressor protein with catenins. Science *262*, 1734-1737.

Tai, J., Cheung, S., Chan, E., and Hasman, D. (2010). Antiproliferation effect of commercial brewed coffees on human ovarian cancer cells in vitro. Nutr. Cancer *62*, 1044-1057.

Takahashi, M., and Wakabayashi, K. (2004). Gene mutations and altered gene expression in azoxymethane-induced colon carcinogenesis in rodents. Cancer Sci. *95*, 475–480.

Tetsu, O., and McCormick, F. (1999). Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. Nature *398*, 422-426.

Thompson, C.B. (1995). Apoptosis in the pathogenesis and treatment of disease. Science *267*, 1456-1462.

Thomsen, L.L., Miles, D.W., Happerfield, L., Bobrow, L.G., Knowles, R.G., and Moncada, S. (1995). Nitric oxide synthase activity in human breast cancer. Br. J. Cancer *72*, 41-44.

Thomsen, L.L., Scott, J.M., Topley, P., Knowles, R.G., Keerie, A.-J., and Frend, A.J. (1997). Selective inhibition of inducible nitric oxide synthase inhibits tumors growth in vivo: studies with 1400W, a novel inhibitor. Cancer Res. *57*, 3300-3304.

Thornton, J.R. (1981). High colonic pH promotes colorectal cancer. Lancet *1*, 1081-1083.

Thun, M.J., Namboodiri, M.M., and Heath, C.W.J. (1991). Aspirin use and reduced risk of fatal colon cancer. N. Engl. J. Med. *325*, 1593-1596.

Tomlinson, I.P.M., and Bodmer, W.F. (1995). Failure of programmed cell death and differentiation as causes of tumors: some simple mathematical models. Proc. Natl. Acad. Sci. USA *92*, 11130-11134.

Topping, D.L., and Clifton, P.M. (2001). Short-chain fatty acids and human colonic function: roles of resistant starch and nonstarch polysaccharides. Physiol. Rev. *81*, 1031-1064.

Trowell. H. (1972). Dietary fibre and coronary heart disease. Rev. Eur. Etud. Clin. Biol. *17*, 345-349.

Tsai, C.H., Hill, M., Asa, S.L, Brubaker, P.L., and Drucker, D.J. (1997). Intestinal growth-promoting properties of glucagon-like peptide-2 in mice. Am. J. Physiol. *273*, 77-84.

Tsujimoto, Y. (1989). Stress-resistance conferred by high level of bcl-2 a protein in human B lymphoblastoid cell. Oncogene *4*, 1331-1336.

Tsukamoto, T., Tanaka, H., Fukami, H., Inoue, M., Takahashi, M., Wakabayashi, K., and Tatematsu, M. (2000). More frequent β-catenin gene mutations in adenomas than in aberrant crypt foci or adenocarcinomas in the large intestines of 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP)-treated Rats. Jpn. J. Cancer Res. **91**, 792–796.

Tutton, P.J.M., and Barkla, D.H. (1980). Neural control of colonic cell proliferation. Cancer *45*, 1172-1177.

Ulshen, M.H., Dowling, R.H., Fuller, C.R., Zimmermann, E.M, and Lund, P.K. (1993). Enhanced growth of small bowel in transgenic mice overexpressing bovine growth hormone. Gastroenterol. *104*, 973-980.

Umar, A., Buermeyer, A.B., Simon, J.A., Thomas, D.C., Clark, A.B., Liskay, R.M., and Kunkel, T.A. (1996). Requirement of PCNA in DNA mismatch repair at a step preceding DNA resynthesis. Cell *87*, 65-73.

Umesalma, S., and Sudhandiran, G. (2011). Ellagic acid prevent rat colon carcinogenesis induced by 1, 2 dimethyl hydrazine through inhibition of AKT-phosphoinositide-3 kinase pathway. Eur. J. Pharmacol. 660, 249-258.

Vane, J.R., Bakhle, Y.S., and Botting, R.M. (1998). Cyclooxygenases 1 and 2. Am. Rev. Pharmacol. Toxicol. *38*, 97-120.

Verma, B., Hucl, P., and Chibbar, R. N. (2008). Phenolic content and antioxidant properties of bran in 51 varieties of wheat. Cereal Chem. *85*, 544-549.

Verma, B., Hucl, P., and Chibbar, R.N. (2009). Phenolic acid composition and antioxidant capacity of acid and alkali hydrolysed wheat bran fractions. Food Chem. *116*, 947-954.

Vogelstein, B., Fearon, E.R., Hamilton, S.R., Kern, S.F., Preisinger, A.C., Leppert, M., Nakamura, Y., White, R., Smits, A.M., and Bos, J.L. (1988). Genetic alterations during colorectal-tumor development. N. Engl. J. Med. *319*, 525-532.

Vuksan, V., Jenkins, A.L, Jenkins, D.J., Rogovik, A.L., Sievenpiper, J.L., and Jovanovski, E. (2008). Using cereal to increase dietary fiber intake to the recommended level and the effect of fiber on bowel function in healthy persons consuming North American diets. Am. J. Clin. Nutr. *88*, 1256-1262.

Waddell, W.R., and Loughry, R.W. (1983). Sulindac for polyposis of the colon. J. Surg. Oncol. *24*, 83-87.

Wallace-Brodeur, R.R., and Lowe, S.W. (1999). Clinical implications of p53 mutations. Cell Mol. Life Sci. *55*, 64-75.

Wang D., Wang, H., Shi, Q., Katkuri, S., Walhi, W., Desvergne, B., Das, S.K., Dey, S.K., and DuBois, R.N. (2004). Prostaglandin E(2) promotes colorectal adenoma growth via transactivation of the nuclear peroxisome proliferator-activated receptor δ. Cancer Cell *6*, 285–295.

Wargovich, M.J., Eng, V.W.S., and Newmark, H.L. (1984). Calcium inhibits the damaging and compensatory proliferative effects of fatty acids on mouse colonic epithelium. Cancer Lett. *23*, 253-258.

Watanabe, K., Reddy, B.S., Weisburger, J.H., and Kritchevsky, D. (1979). Effect of dietary alfalfa, pectin, and wheat bran on azoxymethane-or methylnitrosourea-induced colon carcinogenesis in F344 rats. J. Natl. Cancer Inst. *63*, 141-145.

Watanabe, K., Kawamori, T., Nakatsugi, S., Ohta, T., Ohuchida, S., Yamamoto, H., Maruyama, T., Kondo, K., Ushikubi, F., Narumiya, S., Suqimura, T., and Wakabayashi, K. (1999). Role of the prostaglandin E receptor subtype EP1 in colon carcinogenesis. Cancer Res. *59*, 5093-5096.

Watanabe, K., Kawamori, T., Nakatsugi, S., and Wakabayashi, K. (2000). COX-2 and iNOS, good targets for chemoprevention of colon cancer. BioFactors *12*, 129-133.

Watson, A.J.M., and Pritchard, D.M. (2000). Lessons from genetically engineered animal models VII. Apoptosis in intestinal epithelium: lessons from transgenic and knockout mice. Am. J. Physiol. Gastrointest. Liver Physiol. *278*, G1-G5.

Whelton, A. (1999). Nephrotoxicity of nonsteroidal anti-inflammatory drugs: physiologic foundations and clinical implications. Am. J. Med. *106*, S13-S24.

Wink, D.A., Vodovotz, Y., Laval, J., Laval, F., Dewhirst, M.W., and Mitchell, J.B. (1998). The multifaceted roles of nitric oxide in cancer. Carcinogenesis *19*, 711-721.

Wirfält, E., Midthune, D., Reedy, J., Mitrou, P., Flood, A., Subar, A.F. Leitzmann, M., Mouw, T., Hollenbeck, A.R., Schatzkin, A., and Kipnis, V. (2009). Associations between food patterns defined by cluster analysis and colorectal cancer incidence in the NIH-AARP diet and health study. Eur. J. Clin. Nutr. *63*, 707-717.

Wu, Q.K., Koponen, J.M., Mykkanen, H.M., and Torronen, A.R. (2007). Berry phenolic extracts modulate the expression of p21WAF1 and Bax but not Bcl-2 in HT-29 colon cancer cells. J. Agric. Food Chem. *55*, 1156-1163.

Wu, C.-H., Shih, Y.-W., Chang, C.-H., Ou, T.-T., Huang, C.-C., Hsu, J.-D., and Wang, C.-J. (2010). EP4 upregulation of Ras signaling and feedback regulation of Ras in human colon tissus and cancer cells. Arch. Toxicol. *84*, 731-740.

Yamamoto, H., Imai, K., and Perucho, M. (2002). Gastrointestinal cancer of the microsatellite mutator phenotype pathway. J. Gastroenterol. *37*, 153-163.

Yang, C.S., Landau, J.M., Huang, M.-T., and Newmark, K.L. (2001). Inhibition of carcinogenesis by dietary polyphenolic ciompounds. Annu. Rev. Nutr. *21*, 381-406.

Yoshioka, K., Deng, T.L., Cavigelli, M., and Karin, M. (1995). Antitumor promotion by phenolic antioxidants – Inhibition of AP-1 activity through induction of Fra expression. Proc. Natl. Acad, Sci. USA *92*, 4972-4976.

Yoshioka, K., Kataoka, T., Hayashi, T., Hasegawa, M., Ishi, Y., and Hibasami, H. (2000). Induction of apoptosis by gallic acid in human stomach cancer KATO III and colon adenocarcinoma COLO 205 cell lines. Oncol. Rep. *7*, 1221-1223.

You, B.R., and Park, W. H. (2010). Gallic acid-induced human pulmonary fibroblast cell death is accompanied by increases in ROS level and GSH depletion. Drug Chem. Toxicol. *34*, 38-44.

Young, K.J., and Lee, P.N. (1999). Intervention studies on cancer. Eur. J. Cancer Prev. *8*, 91-103.

Yu, L., Haley, S., Perret, J., Harris, M., Wilson, J., and Qian, M. (2002). Free radical scavenging properties of wheat extracts. J. Agric. Food Chem. *50*, 1619-1624.

Zaidi, N.H., Pretlow, T.P., O'Riordan, M.A., Dumenco, L.L., Allay, E. and Gershon, S.L. (1995). Transgenic expression of human MGMT protects against azoxymethane-induced aberrant crypt foci and G to A mutations in the K-ras oncogene of mouse colon. Carcinogenesis *16*, 451-456.

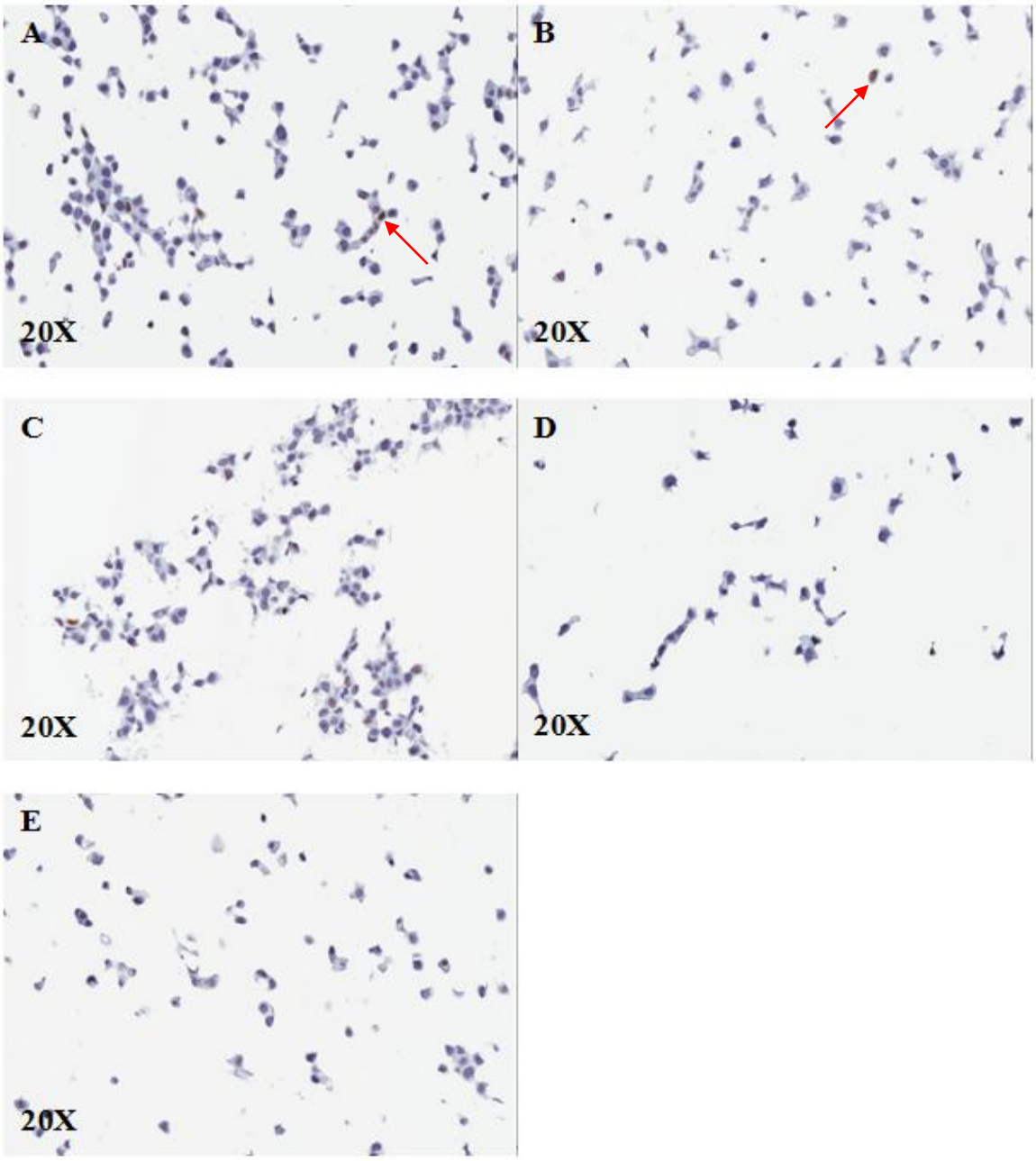
Zetterberg, A., Larsson, O., and Winman, K.G. (1995). What is the restriction point? Curr. Opin. Cell Biol. *7*, 835-842.

Zhang, F., White, R.L., and Neufeld, K.L. (2000a). Phosphorylation near nuclear localization signal regulates nuclear import of adenomatous polyposis coli protein. Proc. Natl. Acad. Sci. USA *97*, 577-582.

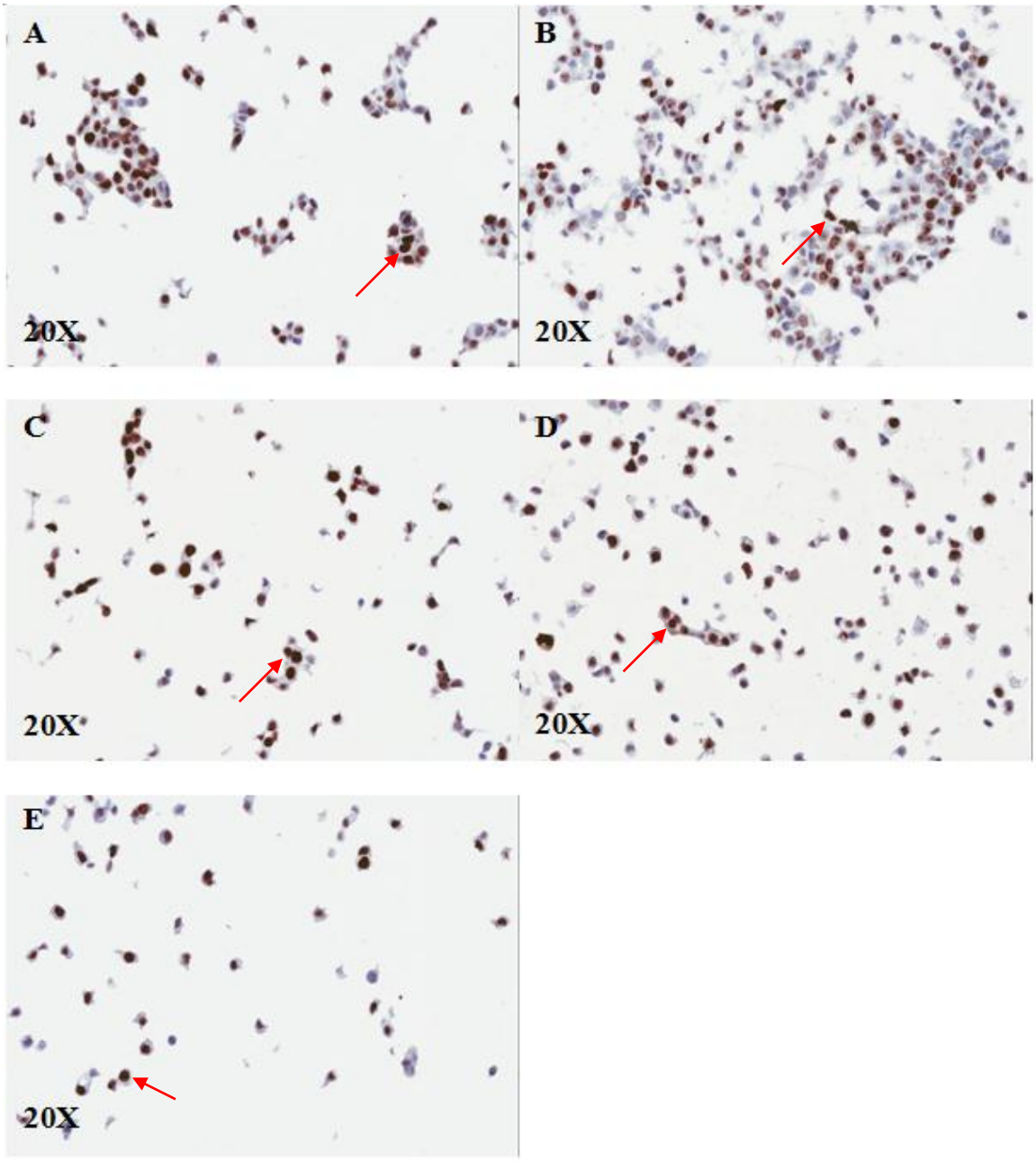
Zhang, L., Yu, J., Park, B.H., Kinzler, K.W., and Vogelstein, B. (2000b). Role of BAX in the apoptotic response to anticancer agents. Science *290*, 989-992.

Zhao, Y., Agarwal, V.R., Mendelson, C.R., and Simpson, E.R. (1996). Estrogen biosynthesis proximal to a breast tumor is stimulated by PGE_2 via cyclic AMP, leading to activation of promoter II of the CYP19 (aromatase) gene. Endocrinology *137*, 5739-5742.

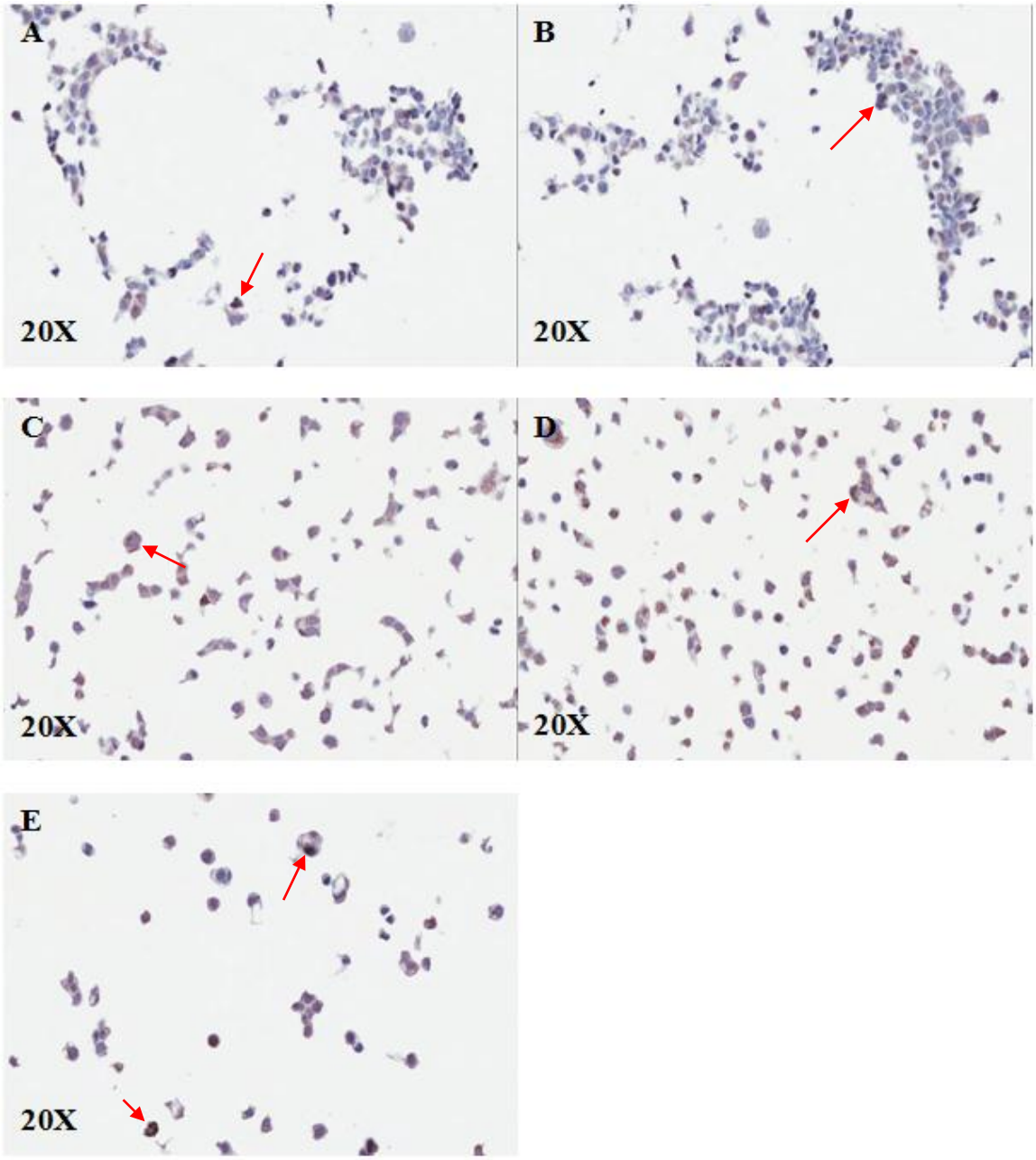
9.0 APPENDIX



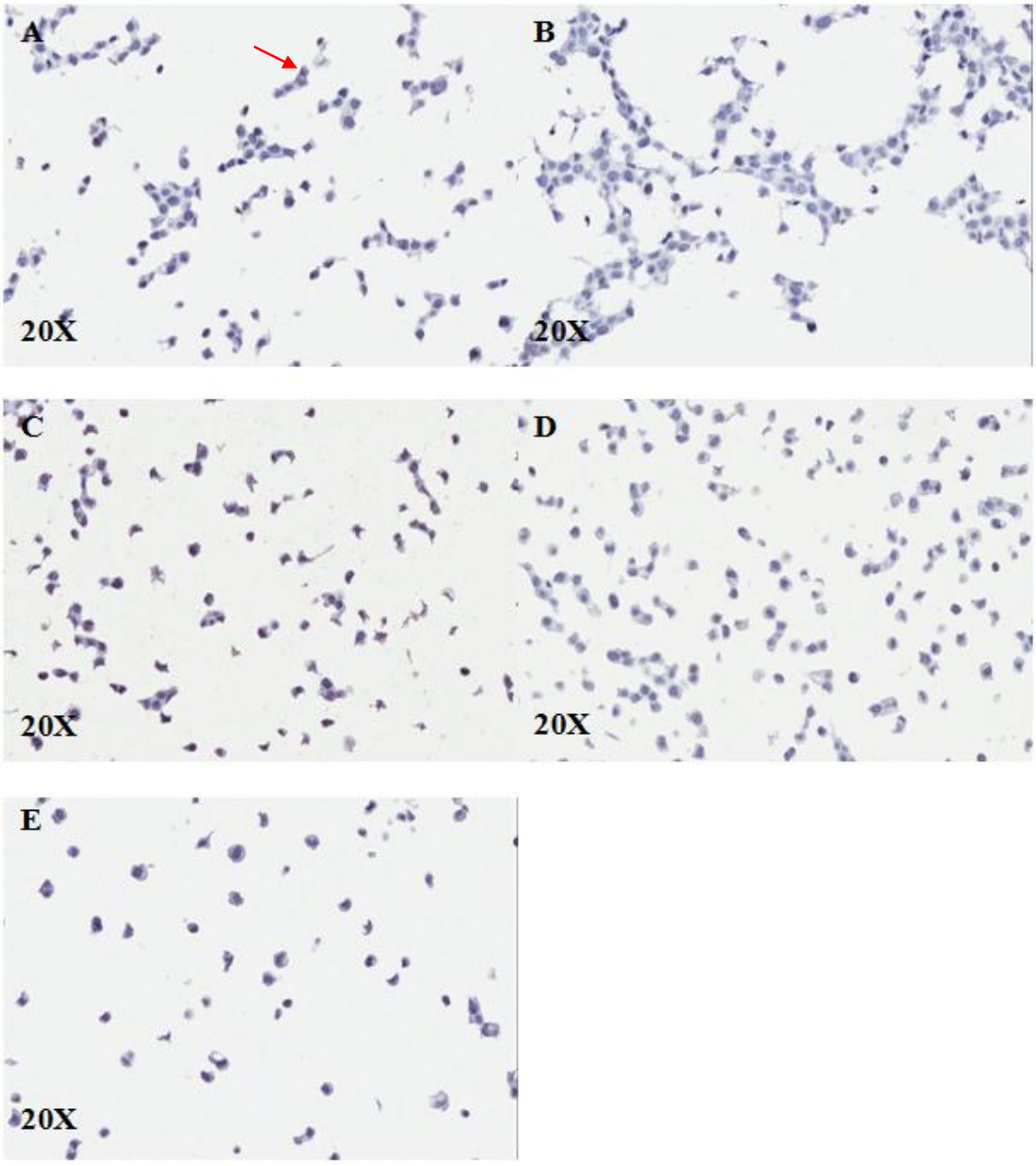
Appendix 1. Representative photographs of histological sections of human colon carcinoma cell line HT-29 treated with chlorogenic acid. The Cyclin D1 proteins in (A) Control or 0 µg/mL, (B) 10 µg/mL, (C) 100 µg/mL, (D) 200 µg/mL, and (E) 400 µg/mL are nucleus staining. Note brown-stained Cyclin D1 proteins (red arrows).



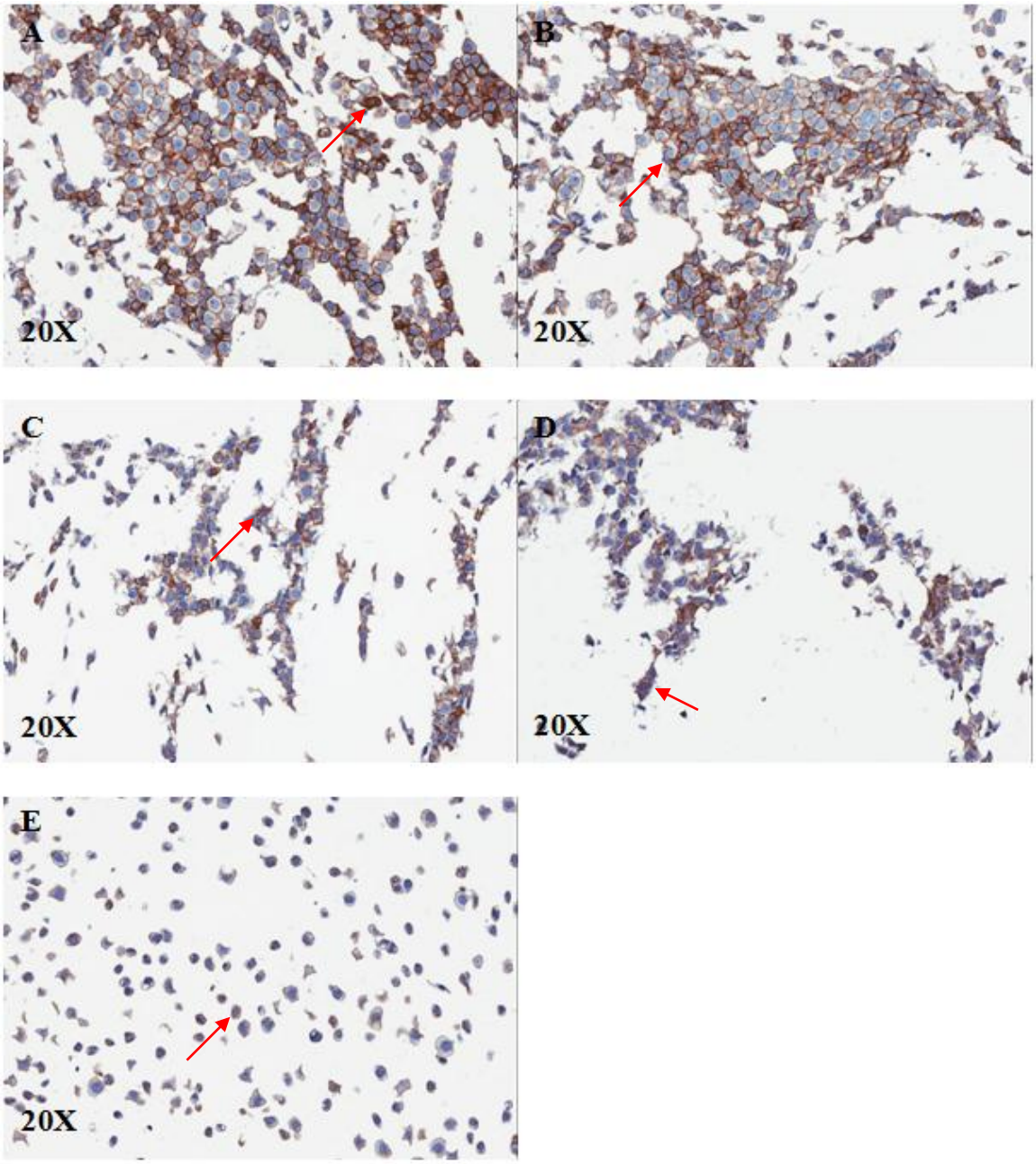
Appendix 2. Representative photographs of histological sections of human colon carcinoma cell line HT-29 treated with chlorogenic acid. The Ki67 proteins in (A) Control or 0 µg/mL, (B) 10 µg/mL, (C) 100 µg/mL, (D) 200 µg/mL, and (E) 400 µg/mL are nucleus staining. Note brown-stained Ki67 proteins (red arrows).



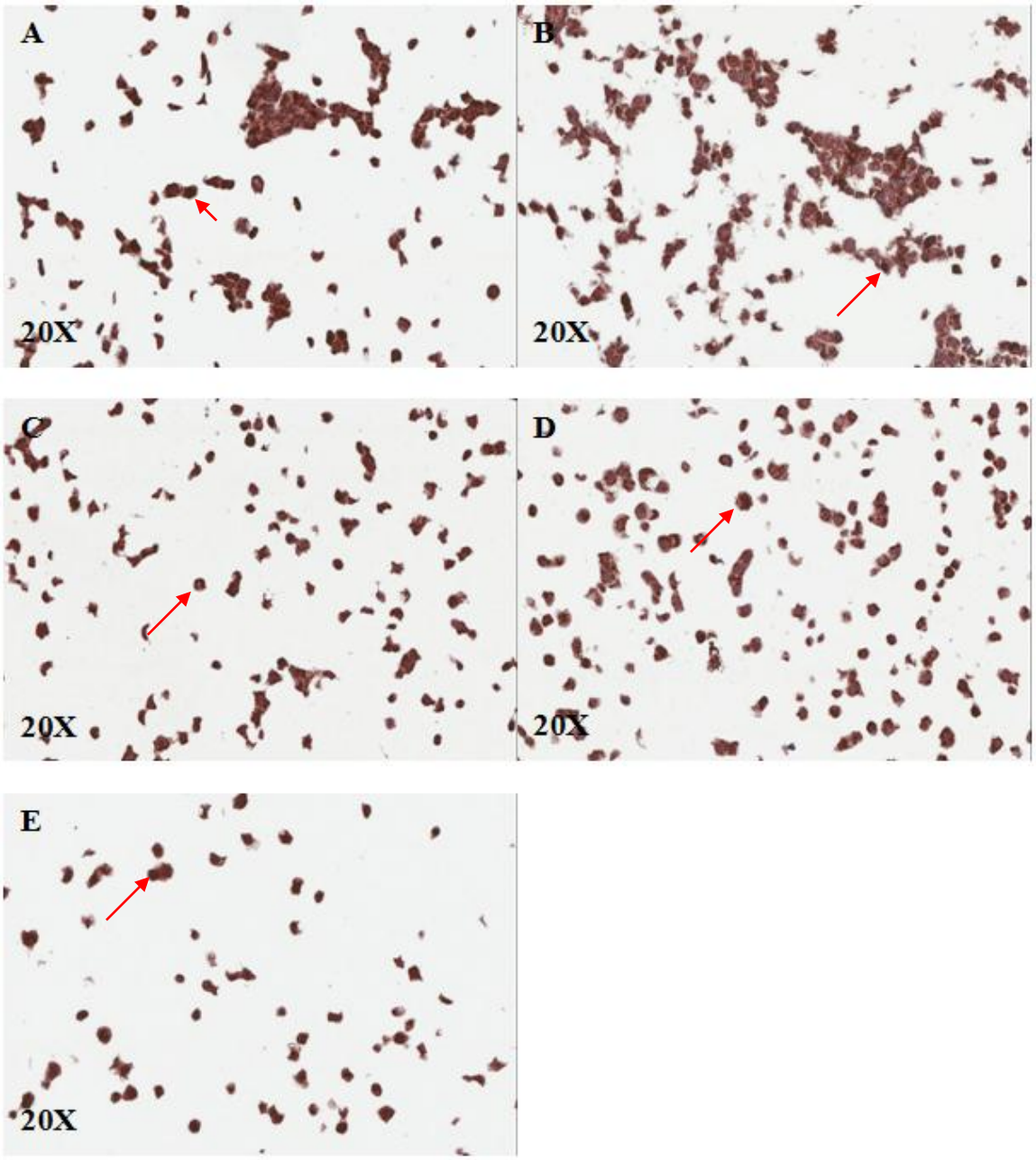
Appendix 3. Representative photographs of histological sections of human colon carcinoma cell line HT-29 treated with chlorogenic acid. The Bax proteins in (A) Control or 0 µg/mL, (B) 10 µg/mL, (C) 100 µg/mL, (D) 200 µg/mL, and (E) 400 µg/mL are cytoplasmic staining. Note brown-stained Bax proteins (red arrows).



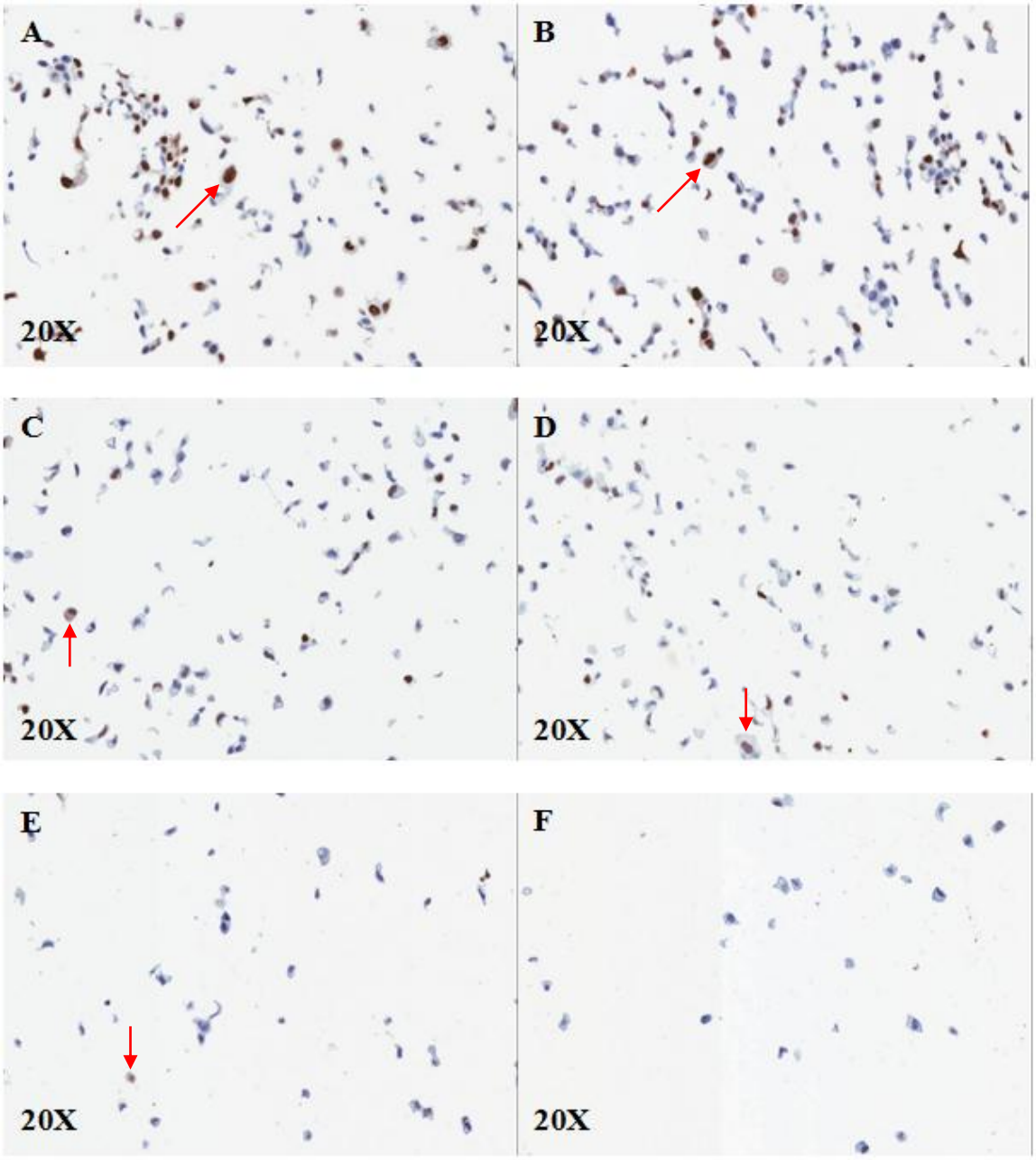
Appendix 4. Representative photographs of histological sections of human colon carcinoma cell line HT-29 treated with chlorogenic acid. The Bcl-2 proteins in (A) Control or 0 µg/mL, (B) 10 µg/mL, (C) 100 µg/mL, (D) 200 µg/mL, and (E) 400 µg/mL are cytoplasmic staining. Note brown-stained Bcl-2 proteins.



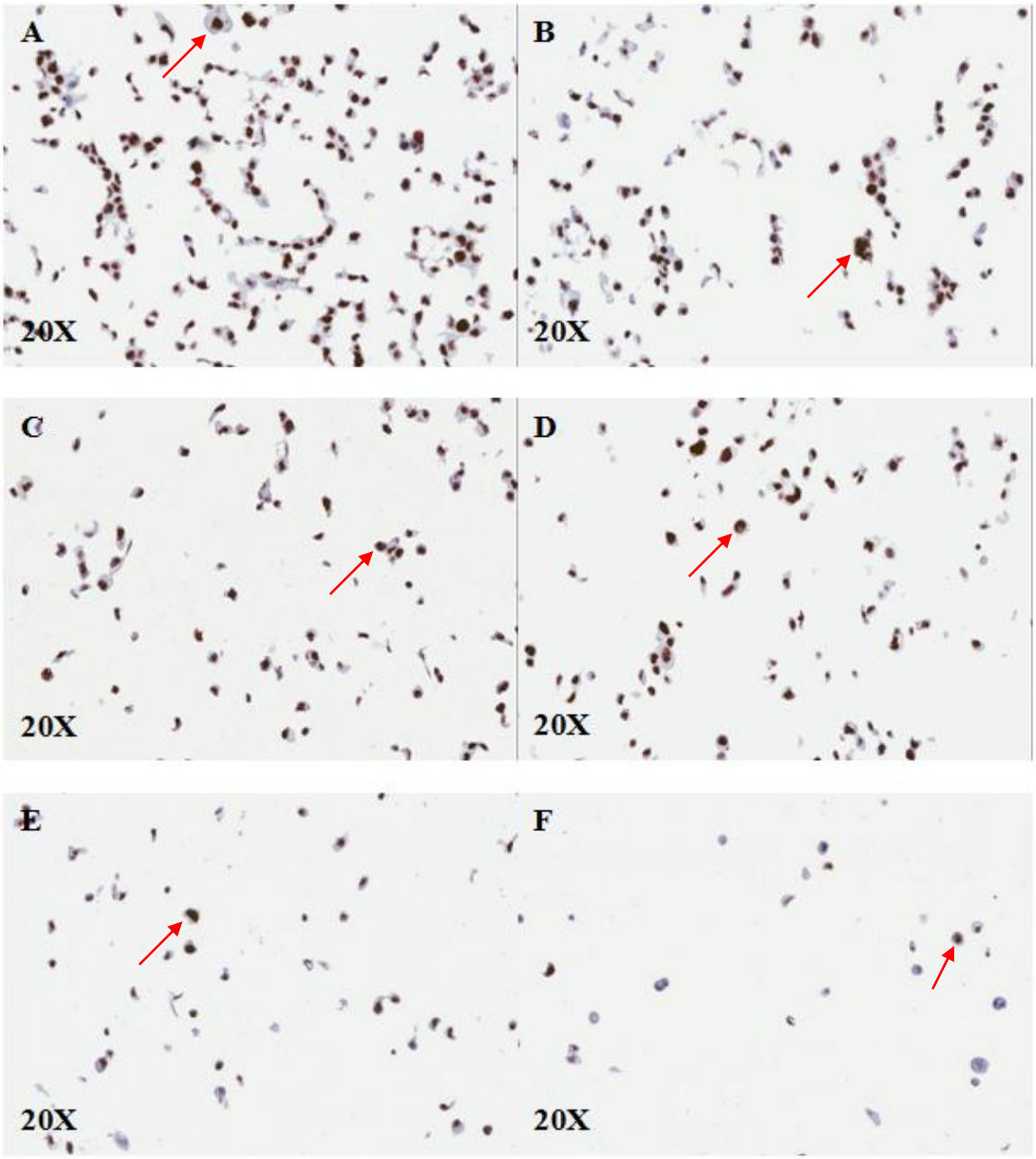
Appendix 5. Representative photographs of histological sections of human colon carcinoma cell line HT-29 treated with chlorogenic acid. The beta-catenin proteins in (A) Control or 0 µg/mL, (B) 10 µg/mL, (C) 100 µg/mL, (D) 200 µg/mL, and (E) 400 µg/mL are cytoplasmic staining. Note brown-stained beta-catenin proteins (red arrows).



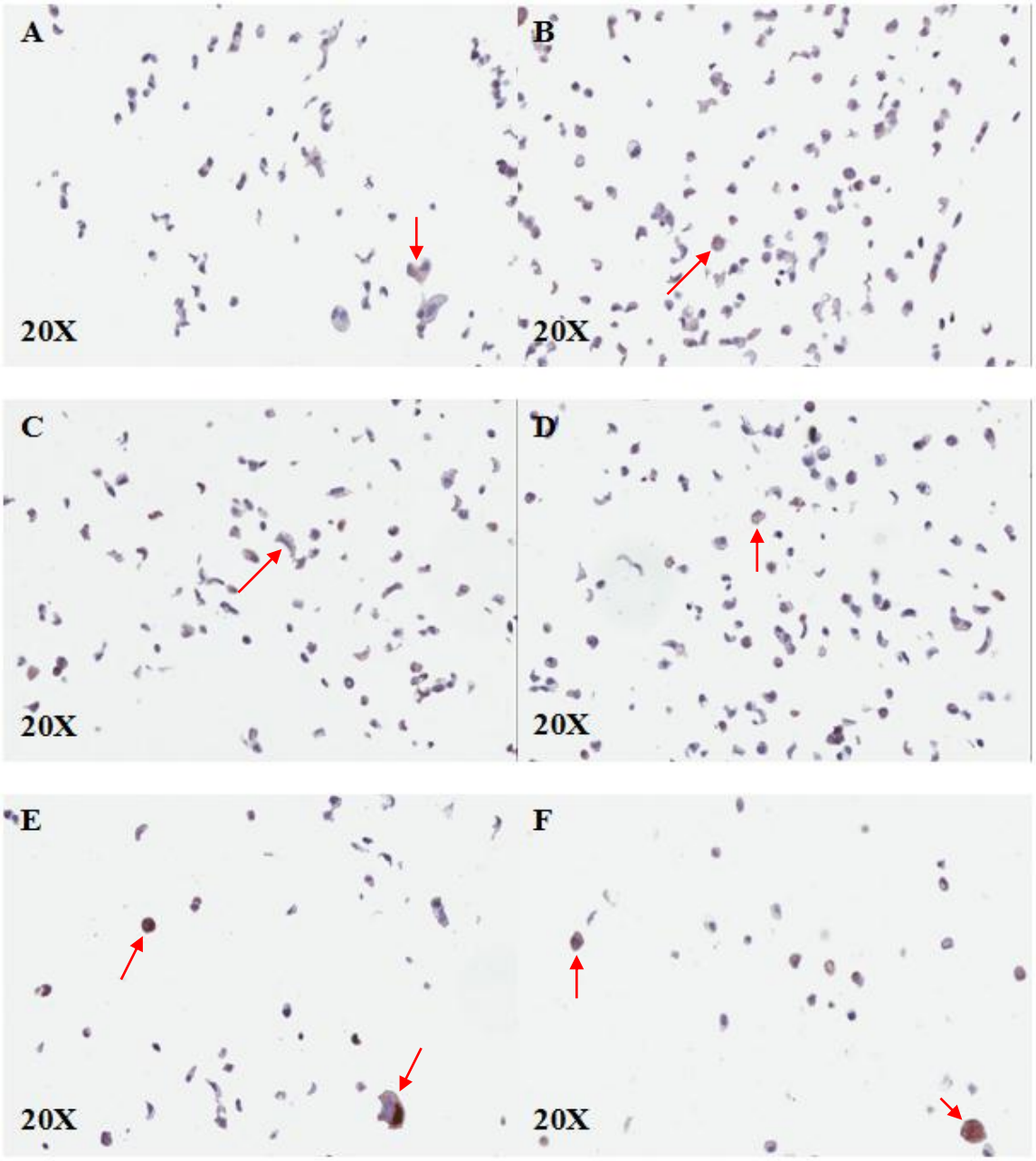
Appendix 6. Representative photographs of histological sections of human colon carcinoma cell line HT-29 treated with chlorogenic acid. The iNOS proteins in (A) Control or 0 µg/mL, (B) 10 µg/mL, (C) 100 µg/mL, (D) 200 µg/mL, and (E) 400 µg/mL are cytoplasmic staining. Note brown-stained iNOS proteins (red arrows).



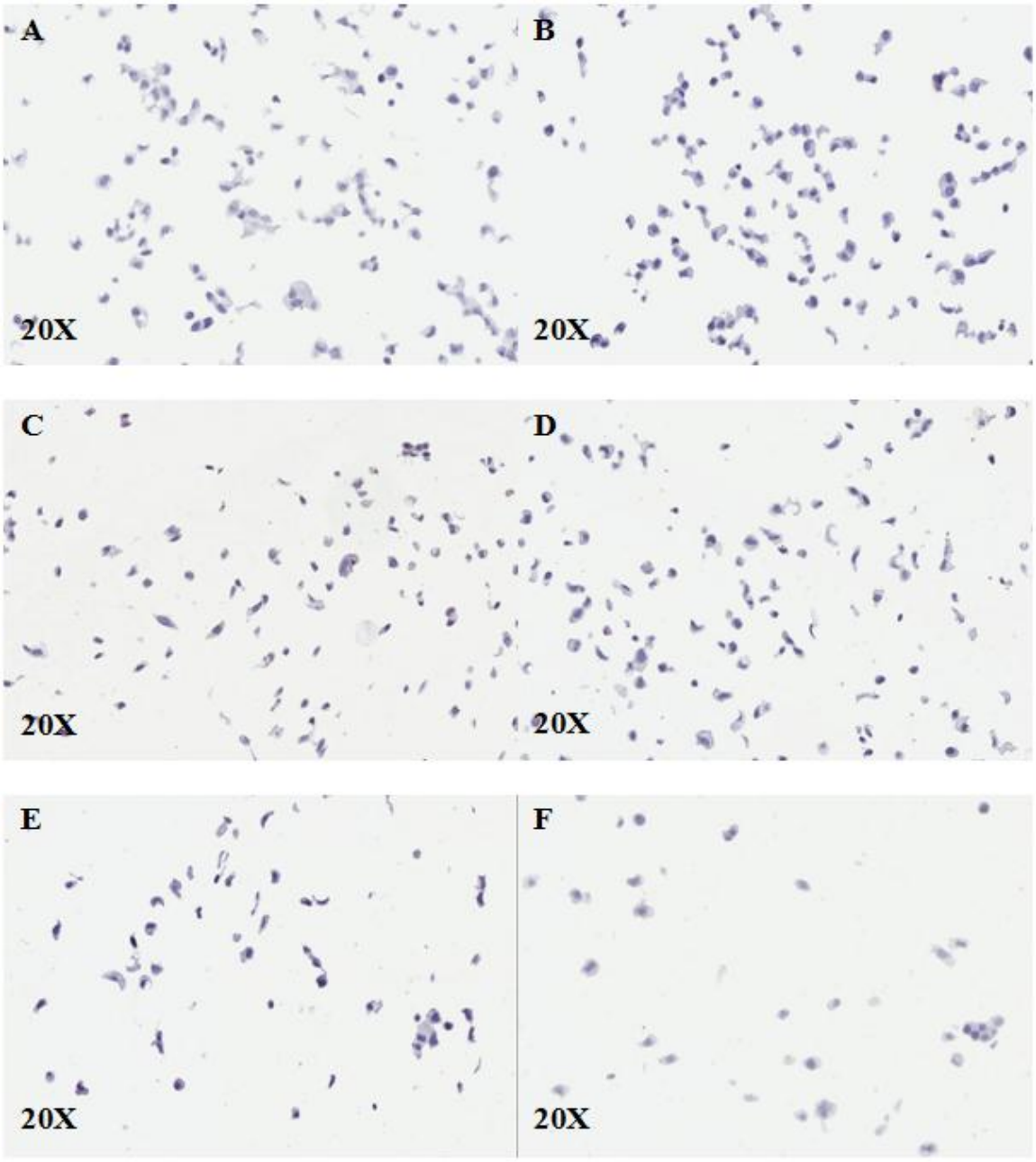
Appendix 7. Representative photographs of histological sections of human colon carcinoma cell line HCT 116 treated with ellagic acid. The Cyclin D1 proteins in (A) Control or 0 µg/mL, (B) 10 µg/mL, (C) 100 µg/mL, (D) 200 µg/mL, (E) 400 µg/mL, and (F) 800 µg/mL are nucleus staining. Note brown-stained Cyclin D1 proteins (red arrows).



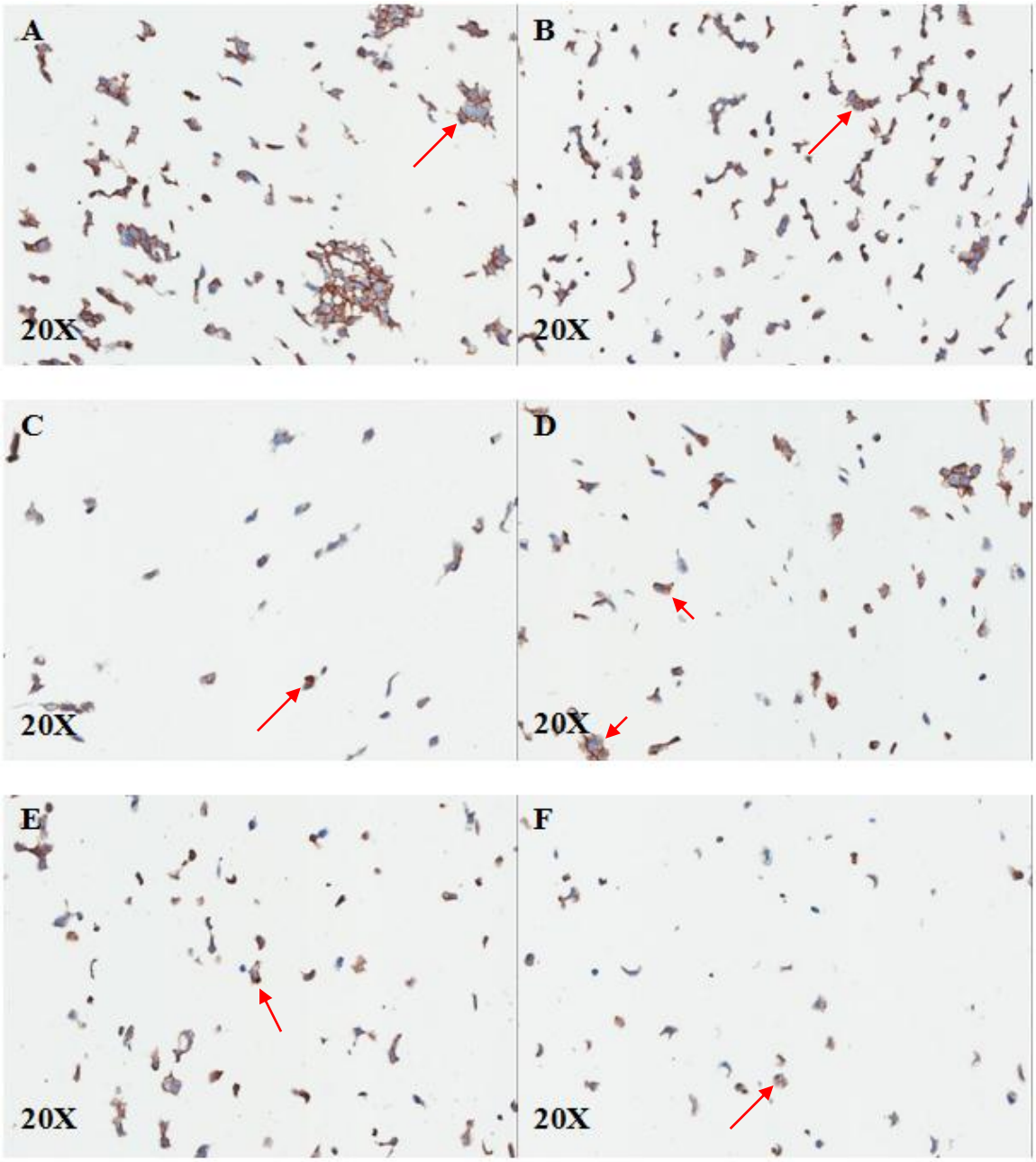
Appendix 8. Representative photographs of histological sections of human colon carcinoma cell line HCT 116 treated with ellagic acid. The Ki67 proteins in (A) Control or 0 µg/mL, (B) 10 µg/mL, (C) 100 µg/mL, (D) 200 µg/mL, (E) 400 µg/mL, and (F) 800 µg/mL are nucleus staining. Note brown-stained Ki67 proteins (red arrows).



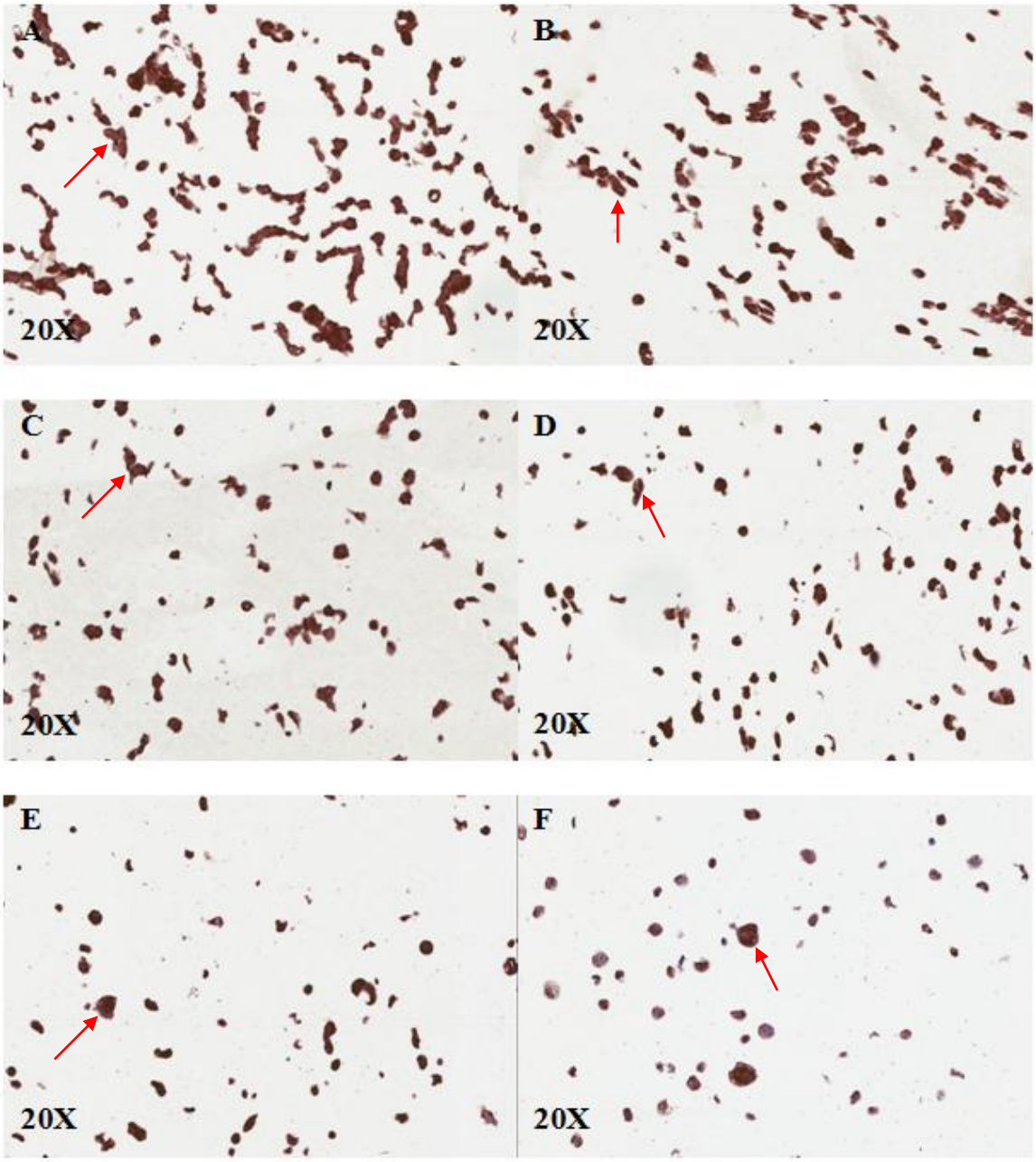
Appendix 9. Representative photographs of histological sections of human colon carcinoma cell line HCT 116 treated with ellagic acid. The Bax proteins in (A) Control or 0 µg/mL, (B) 10 µg/mL, (C) 100 µg/mL, (D) 200 µg/mL, (E) 400 µg/mL, and (F) 800 µg/mL are cytoplasmic staining. Note brown-stained Bax proteins (red arrows).



Appendix 10. Representative photographs of histological sections of human colon carcinoma cell line HCT 116 treated with ellagic acid. The Bcl-2 proteins in (A) Control or 0 µg/mL, (B) 10 µg/mL, (C) 100 µg/mL, (D) 200 µg/mL, (E) 400 µg/mL, and (F) 800 µg/mL are cytoplasmic staining. Note brown-stained Bcl-2 proteins.



Appendix 11. Representative photographs of histological sections of human colon carcinoma cell line HCT 116 treated with ellagic acid. The beta-catenin proteins in (A) Control or 0 µg/mL, (B) 10 µg/mL, (C) 100 µg/mL, (D) 200 µg/mL, (E) 400 µg/mL, and (F) 800 µg/mL are cytoplasmic staining. Note brown-stained beta-catenin proteins (red arrows).



Appendix 12. Representative photographs of histological sections of human colon carcinoma cell line HCT 116 treated with ellagic acid. The iNOS proteins in (A) Control or 0 µg/mL, (B) 10 µg/mL, (C) 100 µg/mL, (D) 200 µg/mL, (E) 400 µg/mL, and (F) 800 µg/mL are cytoplasmic staining. Note brown-stained iNOS proteins (red arrows).

Appendix 13. Relative quantitation of Western Blot of cell treatments from either human colon cancer cell line HT-29 or HCT 116 probed with primary antibody against Bax or Cyclin D1. The numbers indicate the number-fold increase or reduce of each specific immunoreactive band compared with its control, to which a value of 1 was assigned to be equal. "nd", not detectable.

	Polypeptide (Cell line – Phenolic acid used)	Control	10	100	200	400	800
(a)	Bax (HCT 116 – Caffeic acid)	1.00	1.30	1.68	3.51	nd	nd
(b)	Bax (HCT 116 – Ellagic acid)	1.00	1.38	2.27	2.42	2.47	nd
(c)	Bax (HCT 116 – Gallic acid)	1.00	1.05	nd	nd	nd	nd
(d)	Cyclin D1 (HCT 116 – Caffeic acid)	1.00	0.66	0.50	0.46	nd	nd
(e)	Cyclin D1 (HCT 116 – Ellagic acid)	1.00	0.98	0.77	0.49	0.34	nd
(f)	Cyclin D1 (HCT 116 – Gallic acid)	1.00	0.81	nd	nd	nd	nd
(g)	Bax (HT-29 – Caffeic acid)	1.00	1.30	1.66	1.78	nd	nd
(h)	Bax (HT-29 – Chlorogenic acid)	1.00	1.29	2.10	2.08	2.75	3.12
(i)	Bax (HT-29 – Gallic acid)	1.00	1.10	nd	nd	nd	nd
(j)	Cyclin D1 (HT-29 – Caffeic acid)	1.00	0.19	0.00	0.00	nd	nd
(k)	Cyclin D1 (HT-29 – Chlorogenic acid)	1.00	0.63	0.93	0.87	1.11	0.48
(l)	Cyclin D1 (HT-29 – Gallic acid)	1.00	0.71	nd	nd	nd	nd